Crystal Structure of Escherichia coli Ketopantoate Reductase in a Ternary Complex with NADP$^+$ and Pantoate Bound
SUBSTRATE RECOGNITION, CONFORMATIONAL CHANGE, AND COOPERATIVITY

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Ketopantoate reductase (KPR, EC 1.1.1.169) catalyzes the NADPH-dependent reduction of ketopantoate to pantoate, an essential step for the biosynthesis of pantothenate (vitamin B$_5$). Inhibitors of the enzymes of this pathway have been proposed as potential antibiotics or herbicides. Here we present the crystal structure of Escherichia coli KPR in a precipicatry ternary complex with NADP$^+$ and pantoate bound, solved to 2.3 Å of resolution. The asymmetric unit contains two protein molecules, each in a ternary complex; however, one is in a more closed conformation than the other. A hinge bending between the N- and C-terminal domains is observed, which triggers the switch of the essential Lys$_{176}$ to form a key hydrogen bond with the C2 hydroxyl of pantoate. Pantoate forms additional interactions with conserved residues Ser$_{244}$, Asn$_{98}$, and Asn$_{180}$ and with two conservatively varied residues, Asn$_{194}$ and Asn$_{241}$. The steady-state kinetics of active site mutants R31A, K72A, N98A, K176A, S244A, and E256A implicate Asn$_{98}$ as well as Lys$_{176}$ and Glu$_{256}$ in the catalytic mechanism. Isothermal titration calorimetry studies with these mutants further demonstrate the importance of Ser$_{244}$ for substrate binding and of Arg$_{31}$ and Lys$_{72}$ for cofactor binding. Further calorimetric studies show that KPR discriminates binding of ketopantoate against pantoate only with NADPH bound. This work provides insights into the roles of active site residues and conformational changes in substrate recognition and catalysis, leading to the proposal of a detailed molecular mechanism for KPR activity.

Pantothenate (vitamin B$_5$) is the precursor of the 4'-phosphopantetheine moiety of coenzyme A and acyl carrier proteins, which play an important role in metabolism and fatty acid biosynthesis (1–3). The biosynthetic pathway for pantothenate has been elucidated in Escherichia coli and other bacteria and is composed of four enzymes. The first two convert α-ke-toisovalerate to pantoate, then in a separate branch L-aspartate is decarboxylated to produce β-alanine. Finally, pantoate and β-alanine are condensed together to form pantothenate (4, 5). The pathway is similar in plants and fungi, although they appear to use a different route to β-alanine (6). Bioinformatics analysis has identified the pantothenate pathway as a potential antimicrobial target (7). This is supported by recent genetic studies which show that a pantothenate auxotroph of Mycobacterium tuberculosis fails to establish chronic infections in mice (8).

Ketopantoate reductase (KPR, EC 1.1.1.169), encoded by the panE gene, is the second enzyme in the pathway and catalyzes the NADPH-dependent reduction of ketopantoate to pantoate. Previous biochemical studies on the Escherichia coli enzyme established that hydride transfer is stereospecific from the pro-$S$ proton of NADPH to the $si$ face of ketopantoate (Scheme 1A), and the reaction equilibrium favors NADP$^+$ and pantoate formation (9, 10). Steady-state kinetic and inhibition analysis are consistent with a sequential ordered bibi kinetic mechanism (Scheme 1B) in which NADPH binding is followed by ketopantoate binding, and then pantoate release precedes NADP$^+$ release (10). The pH dependence of catalysis is consistent with the involvement of a general acid/base in the catalytic mechanism (9, 10). Site-directed mutagenesis implicated Lys$_{176}$ and Glu$_{256}$ as important for catalysis (9).

The crystal structure of the apoenzyme was solved by Matak-Vinkovic et al. (11) at 1.7 Å of resolution. KPR belongs to the 6-phosphogluconate dehydrogenase superfamily in the SCOP data base (11, 12). Among other enzymes in this superfamily are ace-tyloxyacid isomeroreductase (13), short chain l-3-hydroxyacyl-CoA dehydrogenase (14), D'-pyrroline-5-carboxylate reductase (15), and prephenate dehydrogenase (16). The secondary structure of KPR comprises 13 α-helices and 11 β-strands. The enzyme is monomeric with a molecular mass of 34 kDa and is composed of a coenzyme binding domain and a substrate binding domain separated by a large cleft. The N-terminal domain has an αβ Rossmann-type fold featured in many nucleotide-binding proteins, with a glycine-rich region (GCCGALG$^{12}$) for coenzyme recognition (17). The C-terminal substrate binding domain is composed of 8 α-helices and has a core of two long antiparallel helices, which is a common motif within the superfamily.

* This work was supported by the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Structure of KPR Ternary Complexes

A

E NADPH ketopantoate pantoate NADP*  

B

NADPH ketopantoate pantoate NADP*  

SCHEME 1. A, ketopantoate is reduced by NADPH, with relevant atoms numbered. The pro-S hydrate of NADPH is transferred to the si face of ketopantoate. B, sequential ordered biiibi kinetic mechanism.

More recently, we solved the crystal structure of KPR in complex with NADP+ to 2.1 Å of resolution (18). Surprisingly, it was found to be in the same open conformation as the structure of apoenzyme. The cofactor is bound in an extended conformation encompassing the active site cleft, with the nicotinamide ring adopting a syn conformation. Analysis of this structure led to the proposal that Lys176 could act as the general acid/base for catalysis. This required a significant change in the conformation of the lysine to switch to an “active” state where it can hydrogen bond to the substrate. However, there was no direct structural evidence for this conformational change of Lys176. Elucidation of the atomic details of substrate binding and of the catalytic mechanism required crystal structures of the ternary complex, which has yet been unsuccessful (18, 19).

Here we present the three-dimensional structure of E. coli KPR in a ternary complex with NADP+ and pantoate, solved to 2.3 Å of resolution. Two protein molecules were found in the asymmetric unit, each with NADP+ and pantoate bound. One complex is in an open form, and the other is in a closed form, demonstrating that a hinge bending domain closure occurs. Calorimetric and kinetic studies on ternary complexes and engineered mutants provide additional insights into the molecular details of catalysis.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma Aldrich unless otherwise stated. Solutions for crystallography were from Qiagen Nextel. Ketopantoate and pantoate were prepared by hydrolysis of their respective lactones with NaOH as described elsewhere (20).

Protein Expression and Purification—Wild-type (WT) KPR and site-directed mutants of KPR were expressed from a pRSETA plasmid, which adds 17 amino acid residues (MRGSHHHHHHGLVPRGS) to the N terminus of the recombinant protein, including a His6 tag. Expression, single-step affinity purification, and characterization of His6-KPR proteins were conducted as previously described (21). Purified proteins were buffer-exchanged in 50–100 mM HEPES-HCl, pH 7.6, using a HiPrep 26/10 Desalting column (GE Healthcare) and used for calorimetric and kinetic studies. For crystallization studies, the WT protein was further purified by size exclusion chromatography using a HiLoad 26/60 Super-Dex 200 gel filtration column (GE Healthcare). The column was eluted using 50 mM HEPES-HCl, pH 7.6, at a flow rate of 1.0 ml min⁻¹. His6-KPR runs as a monomer in gel filtration (apparent molecular mass of ~32 kDa). Protein concentration was determined by A₂₈₀ with an extinction co-efficient of 62,650 M⁻¹ cm⁻¹ obtained from amino acid analysis (Protein and Nucleic Acid Chemistry Facility, Cambridge). The integrity and purity (>95%) of protein samples were determined by SDS-PAGE and electrospray mass spectrometry.

Crystallization of His6-KPR—Crystal trials were carried out using vapor diffusion by the hanging drop vapor-diffusion technique using a protein concentration of 15–30 mg/ml. Before crystallization KPR samples were incubated at 20 °C with 2 mM NADP+ and 10 mM pantoate for 10 min. Single rhombohedrally shaped crystals of KPR were obtained in drops made of 2 μl of protein solution mixed with an equal volume of well solution composed of 35% v/v dioxane. To facilitate data collection at 100 K, these crystals were cryo-protected using the well solution in the presence of 20% v/v 2-methyl-2,4-pentanediol.

X-ray Data Collection, Structure Determination, and Refinement—X-ray data to 2.3 Å of resolution were collected at European Synchrotron Radiation Facility synchrotron, beam station ID14.4 (Grenoble, France). The diffraction data statistics are shown in Table 1. The data were scaled, merged, and reduced using HKL suite (22). The crystal belongs to the primitive tetragonal lattice, space group P4₁2₁2, with cell parameters a = b = 101.7 Å and c = 171.2 Å. Analysis of the Matthews coefficient indicated the presence of two molecules in the asymmetric unit of the crystal. This corresponds to a solvent content of 58%. Calculation of the self-rotation function showed the presence of a non-crystallographic 2-fold axis suggesting a dimeric arrangement of the two molecules of KPR. The structure was solved by molecular replacement using AMoRe from the CCP4 suite (23–25). First, the KPR-NADP⁺ complex (hoKPR, PDB code 1YJQ (18)) was used as the molecular replacement search probe, and one clear solution (monomer A) was found. However, the second solution was not present in the top 10 peaks of the rotation function. The second molecule (monomer B) was subsequently found using molecular replacement with apoKPR (PDB code 1KS9 (11)) as the search probe. Before calculation, the probes were edited by removing all non-protein atoms. The apo- and holoprotein probes were placed in the crystal cell according to the rotational and translational parameters obtained. The resulting correlation coefficient and R cryst between observed and calculated structure factor amplitudes were 54.8 and 49.2%, respectively. The non-crystallographic 2-fold relationship between the two
Crystallographic data collection and refinement statistics

| Data collection                      | ESRF, European Synchrotron Radiation Facility. |
|-------------------------------------|------------------------------------------------|
| X-ray source                        | ESRF, ID14.4                                    |
| Space group                         | P4 2 2                                          |
| Cell parameters, Å (α = β = γ = 90°) | a = b                                          |
|                                   | c                                               |
| Wavelength, Å                      | 0.977                                           |
| Resolution range, Å (outer shell)   | 50.0-2.30 (2.35-2.30)                           |
| No. of unique reflections          | 40,629                                         |
| Multiplicity                       | 9.3                                            |
| Rmerge, % (outer shell)             | 7.3 (42.5)                                      |
| Average I/σ(I)                     | 12.8                                           |
| % Reflections with I/σ(I) > 3       | 86.6 (64.5)                                     |
| Mosaicity, °                       | 0.33                                           |
| Wilson B, Å²                        | 38.8                                           |

Refinement

| Resolution range, Å              | 43.9-2.3                                      |
| Rcryst, %                       | 15.6                                          |
| Rfree, %                        | 21.5                                          |
| Number of reflections           | 36,501                                        |
| Working set                     | 2,032                                         |
| Test set                        | 36,501                                        |
| Number of protein residues      | 587                                           |
| Water molecules                 | 508                                           |
| Dioxane                         | 4                                             |
| Acetate                         | 1                                             |
| NADP⁺ (NAP)                     | 2                                             |
| Pantoate (PAF)                  | 2                                             |

Model quality

| Estimated co-ordinate error, Å² | 0.19                                          |
| Ramachandran plot, %            | 93.9                                          |
| Most favored                    |                                                |
| Generously allowed              | 0                                             |
| Disallowed                      | 0                                             |
| r.m.s.d. bonds, Å               | 0.014                                         |
| r.m.s.d. angles, °              | 1.45                                          |
| Overall mean B, Å²              | 36.3                                          |

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KPR molecules in the asymmetric unit was consistent with the results of the self-rotation function.

After molecular replacement, rigid body refinement was performed by splitting each of KPR monomers into two separate rigid bodies corresponding to the N- and C-terminal domains (residues 1–169 and 170–291, respectively). Simulated annealing was carried out using CNS (26), improving the Rcryst and Rfree factors to 27.8 and 32.3%, respectively, and initial electron density maps were produced. The structure was refined using successive rounds of manual rebuilding in Coot version 0.0.33 (27) and maximum likelihood refinement using Refmac 5 from the CCP4 suite (25, 28) until no unexplained electron density remained, and the Rcryst and Rfree values converged at 15.6 and 21.5%, respectively (Table 1). The final structure includes two molecules of KPR (monomer A, residues 0–292; monomer B, residues 0–293), each in complex with one molecule of NADP⁺ and one molecule of pantoate. In addition, four molecules of dioxane and one molecule of acetate have been included in the modeled solvent. Coordinates for the non-protein molecules were obtained from the HiCUP database (29), and some refinement libraries were obtained using monomer library Sketcher (CCP4). The following residues were built with double conformations: Arg170, Glu209, and Glu240 in monomer A; Tyr206, Ser77, Arg170, Glu172, Glu206, Glu209, Glu216, and Arg217 in monomer B. None of the above residues is near the active site.

Site-directed Mutagenesis—Site-directed mutants of KPR corresponding to R31A, K72A, N98A, K176A, S244A, and E256A were made with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions using pRS ETA-panE as a template. The primers used were from Sigma Genosys and are listed in supplemental Table S1. Each mutation was verified by DNA sequencing (Lark Technologies), and the molecular masses of the recombinant His-tagged mutant enzymes were determined using electrospray mass spectrometry.

UV-based Kinetic Analysis—Assays were carried out on a Biotech Powerwave XS plate reader equipped with KC4 Version 3.2 Biotech instrument software using a 96-well plate with a total volume of 0.2 ml in each well. Enzyme activity was assayed at 25 °C by monitoring the decrease in absorbance at 340 nm over time due to the enzyme-catalyzed oxidation of NADPH to NADP⁺ (ε340 nm for NADPH = 6220 M⁻¹ cm⁻¹). A typical reaction for WT KPR contained 100 mM HEPES-HCl buffer, pH 7.6, 2–100 μM NADPH, 0.02–1 mM ketopantoate, and 1–5 nM enzyme. The concentration of active WT and mutant forms of KPR was accurately determined by performing ITC titrations with the cofactor (see below). The reaction was initiated by addition of ketopantoate. Measurements were obtained at least in duplicate. Initial rates were obtained from the data corresponding to the conversion of the first 10% of substrate. Data were fitted to the Michaelis-Menten rate equation using the GraFit software (Version 5.0.6, Erithacus Software Ltd). Isothermal Titration Calorimetry—Titrations were performed at 27 °C on OMEGA and VP-ITC iso thermal titration calorimeters (Microcal, Inc.). Thermodynamic analysis of binary complexes were performed as previously described (18, 21). For thermodynamic analysis of ternary complexes, the second ligand was titrated to the binary complex formed after completion of titrations of KPR with the first ligand. To ensure near saturation of the enzyme, the final concentration of the first ligand was ~10×Kd in the case of ketopantoate and pantoate and 2–3× the enzyme concentration in the case of NADPH and NADP⁺. Concentrations of cofactors were measured by UV-visible spectrophotometry using the extinction coefficients 6,220 M⁻¹ cm⁻¹ at 340 nm for NADPH (30) and 18,000 M⁻¹ cm⁻¹ at 260 nm for NADP⁺ (31). The heat change accompanying the titration was recorded as differential power by the instrument and determined by integration of each peak obtained. Titrations of ligand to buffer were performed to allow for baseline corrections. The corrected heat change was then fitted using nonlinear least-squares minimization to obtain the dissociation constants, Kq, the enthalpy of binding, ΔH, and the stoichiometry, n. A stoichiometry of 1, determined from titrations with NADP(H) under high c values conditions, was fixed during curve-fitting of data obtained for ketopantoate and pantoate under low c values (21).

RESULTS

Previous attempts to obtain ternary complexes of KPR were made with the aim of forming “dead-end” complexes by co-crystallization with either NADP⁺ and ketopantoate (18) or...
with NADPH and pantoate (19). In both cases crystals were obtained at pH 4.5; however, analyses of the crystal structures revealed no evidence of substrate or product bound. In the first structure, the binary complex with NADP$^+$ alone was obtained (18). Binding of ketopantoate was not observed due to its low affinity for the KPR$^{NADP^+\cdot}$ complex under the experimental conditions of crystallization. In the second structure the low pH of crystallization resulted in the chemical degradation of NADPH to 2'-phospho-ADP-ribose (19). Electron density corresponding to 2'-phospho-ADP-ribose was observed in the active site, whereas no bound pantoate could be observed. These results clearly indicated that crystallization of the enzyme at low pH was not suitable for obtaining ternary complexes. Therefore, the formation of ternary complexes at the more physiologically relevant pH range of 7.0–8.0 was investigated.

**Kinetic and Thermodynamic Studies of Ternary Complexes**

Kinetic analysis of the KPR-catalyzed reaction provided interesting results. At pH 7.5, the enzyme rapidly turns over in the forward direction using NADPH and ketopantoate as substrates, with kinetic parameters $k_{cat} = 25 \text{ s}^{-1}$, $K_m$(NADPH) = 7 $\mu M$, and $K_m$(ketopantoate) = 30 $\mu M$ (21) (see also Table 4). In contrast, it was not possible to detect the reverse reaction at pH 7.5, even using up to millimolar concentrations of NADP$^+$ and pantoate and 20 $\mu M$ enzyme. The failure to detect any catalytic activity at pH 7.5 using NADP$^+$ and pantoate as substrates is consistent with the large apparent equilibrium constant,

$$K_{eq} = \frac{[\text{NADP}^+][\text{pantoate}]}{[\text{NADPH}][\text{ketopantoate}]} = 676 \quad (\text{Eq. 1})$$

measured by Zheng and Blanchard under identical conditions (10). Further evidence for the stability of the Michaelis-Menten ternary complex KPR-NADP$^+\cdot$pantoate at pH 7.5 came from NMR spectroscopy. Simultaneous binding of NADP$^+$ and pantoate to KPR was detected in a $^1H$ WaterLOGSY NMR experiment (18, 32), and no formation of NADPH or ketopantoate was observed (data not shown), suggesting the ternary complex could be formed under thermodynamic equilibrium.

The formation of ternary complexes of KPR with coenzyme and substrate molecules was investigated by ITC at 27 °C. Fig. 1

**FIGURE 1.** Thermodynamic analyses of formation of binary and ternary complexes of E. coli KPR with NADP$^+$ and pantoate by ITC. Integrated data, corrected for the heat of dilution, are shown from titrations at 27 °C of 50 $\mu M$ His$_6$-KPR with 8 mM pantoate (A) and with 0.7 mM NADP$^+$ (B). The resulting binary complexes were further titrated with 0.7 mM NADP$^+$ (C) and with 8 mM pantoate (D). The line represents the least-squares fit to the single-site binding model by the ORIGIN program.

Structure of KPR Ternary Complexes

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VOLUME 282 • NUMBER 11 • MARCH 16, 2007
TABLE 2
Thermodynamic parameters of cofactor and substrate binding to form binary and ternary complexes with E. coli KPR
All ITC titrations were performed at 27 °C in 100 mM HEPES HCl pH 7.6. Errors quoted are those returned by Origin on the curve fitting.

| Ligand in syringe | Protein       | $K_d$ (µM) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $-T \Delta S$ (kcal/mol) |
|------------------|---------------|------------|-----------------------|-----------------------|--------------------------|
| Pantoate         | ApoKPR        | 270 ± 10   | -4.91 ± 0.02          | -8.1 ± 0.2            | 3.2 ± 0.2                |
|                  | KPR-NADP$^+$  | 160 ± 10   | -5.22 ± 0.04          | -5.5 ± 0.1            | 1.8 ± 0.1                |
|                  | KPR-NADPH     | 1100 ± 30  | -4.07 ± 0.01          | -3.2 ± 0.1            | 4.0 ± 0.1                |
| NADP$^+$         | ApoKPR        | 5.8 ± 0.8  | -7.21 ± 0.08          | -6.3 ± 0.3            | -10.5 ± 0.1              |
|                  | KPR-pantoate  | 9.4 ± 1.2  | -6.91 ± 0.07          | 16.3 ± 0.3            | -3.9 ± 0.3               |
| Ketopantoate, $K_m = 30$ µM | ApoKPR      | 6.2 ± 0.3  | -7.16 ± 0.03          | 3.3 ± 0.1             | 2.5 ± 0.1                |
|                  | KPR-NADP$^+$  | 5300 ± 70  | -3.13 ± 0.01          | -5.6 ± 0.1            | -6.4 ± 0.1               |
|                  | KPR-NADPH     | 8100 ± 300 | -2.88 ± 0.02          | 3.5 ± 0.1             | -6.6 ± 0.1               |
| NADPH, $K_m = 7$ µM | ApoKPR      | 0.26 ± 0.11| -9.1 ± 0.3            | -3.1 ± 0.1            | -6.0 ± 0.4                |
|                  | KPR-pantoate  | 1.4 ± 0.6  | -8.1 ± 0.3            | 1.1 ± 0.1             | -9.2 ± 0.3               |
|                  | KPR-ketopantoate | 0.11 ± 0.01| -9.1 ± 0.3            | -3.1 ± 0.1            | -6.0 ± 0.4                |

*$^a$ From Lobley et al. (18).
*$^b$ From Ciulli et al. (21).

FIGURE 2. The structure of the KPR-NADP$^+$-pantoate ternary complex. The two monomers present in the asymmetric unit are related by a non-crystallographic 2-fold axis. Monomer A (shown in green, right) is in an open form, whereas monomer B (shown in cyan, left) is in a closed form. The secondary structure elements are labeled in monomer B. NADP$^+$ and pantoate are shown as sticks (yellow, carbon; blue, nitrogen; red, oxygen; orange, phosphorus). Figs. 2, 3, 4, and 6 were generated and rendered using Pymol Version 0.99 (45).

shows a thermodynamic cycle for the formation of the ternary complex KPR-NADP$^+$-pantoate and the corresponding ITC binding curves to form binary (Fig. 1, A and B) and ternary (Fig. 1, C and D) complexes. The $K_d$ of NADP$^+$ and pantoate from the binary complexes were 6 and 270 µM (Table 2). The above $K_d$ values are similar to those from the ternary complex ($K_d$ of 9 and 160 µM), thus excluding cooperativity between NADP$^+$ and pantoate binding. However, the relative enthalpic and entropic terms changed significantly between binary and ternary complexes. For both pantoate and NADP$^+$, formation of the binary complex is exothermic ($\Delta H$ of $-8.1$ and $-3.2$ kcal/mol; see also Fig. 1, A and B), whereas formation of the ternary complex is endothermic ($\Delta H$ of 1.8 and 6.3 kcal/mol) and driven by favorable $-T \Delta S$. Such large and compensating changes in $\Delta H$ and $-T \Delta S$ (about 10 kcal/mol for each ligand) are often associated with protein conformational changes or protonation events coupled to the formation of the protein-ligand complex (33). Similarly, compensating enthalpic and entropic changes were observed for the formation of the dead-end KPR-NADP$^+$-ketopantoate ternary complex relative to the binary complexes, with no significant changes in $K_d$ (Table 2). However, analysis of ternary complexes with NADPH showed evidence of cooperativity between cofactor and substrate. Binding of NADPH decreases the affinity of pantoate for the enzyme by >4-fold (Table 2). In contrast, ketopantoate binding to the KPR-NADPH ($K_m = 30$ µM) is significantly stronger than that to apoKPR ($K_m = 5$ mM), suggesting that it is enhanced by the presence of NADPH.

Crystallization and Structure Determination—Based on the results of the kinetic and thermodynamic studies of ternary complexes, we attempted to co-crystallize KPR in the presence of saturating amounts of NADP$^+$ and pantoate at pH 7.5. As a result of a sparse matrix screening of a solution containing 0.5–1 mM His$_6$-KPR, 2 mM NADP$^+$, 10 mM pantoate in 50 mM HEPES-HCl, pH 7.5, single rhombohedrally shaped crystals were identified in 35% v/v dioxane (solution 51 of The Classics Suite, Nextal Qiagen). Attempts to crystallize apoKPR or binary complexes of KPR under identical conditions were unsuccessful. Larger crystals were obtained from drops set up by mixing 2 µl of protein solution with 2 µl of this solution. The crystals diffracted to 2.3 Å of resolution. The crystal structure of the KPR-NADP$^+$-pantoate complex was solved using molecular replacement in AMoRe (23, 24). Two molecules of KPR were found in the asymmetric unit of the crys-
The 2Fo – Fc and Fo –Fc electron density maps clearly showed the presence of NADP⁺ and pantoate in the active site cleft of both protomers (Fig. 3).

Overall Crystal Structure—The two molecules of KPR are arranged as a dimer and are related to each other by a non-crystallographic 2-fold axis (Fig. 2). The dimeric interface comprises α5 and β11 at the end of the N-terminal domain and α9 of the C-terminal domain. This dimeric arrangement is likely to be due to crystallographic packing, as the biologically functional state of KPR is monomeric (10), and the asymmetric units of all previous crystal structures consist of one protein monomer (11, 18, 19). One complex (monomer B) is in a more closed form than the other (monomer A). Analysis of main chain atoms r.m.s.d. upon superposition over the N- and C-terminal domain is consistent with a movement between the two domains (see supplemental Table S2). Closer inspection reveals the conformational change is a hinge bending, i.e. a motion perpendicular to the plane of the domain interface (see supplemental Movie S1).

Hinge Bending; Comparison with Apo- and HoloKPR—Co-factor-induced hinge bending domain closure has been observed for several dehydrogenases, including alcohol dehydrogenase (34), formate dehydrogenase (35), glutamate dehydrogenase (36), diaminopimelate dehydrogenase (37), and aspartate-β-semialdehyde dehydrogenase (38). It was, therefore, surprising that the crystal structure of the KPR-NADP⁺ complex compared with apoKPR revealed no evidence of a hinge bending induced by the cofactor (18). Fig. 4 shows a superposition over the main chain atoms of the N-terminal domain and conformational changes of the protein backbone in the C-terminal domain between apo- and holoKPR (Fig. 4A), holo- and monomer A (Fig. 4B), and monomer A and monomer B (Fig. 4C). The pairwise r.m.s.d. values of the C-terminal domain main chain atoms are 1.56 Å (apo/holo), 1.85 Å (holo/monomer A), and 3.45 Å (monomer A/monomer B), suggesting the conformational change is more pronounced along the reaction coordinates (Table 3).

An analysis of the conformational changes between these pairs of structure using the program DynDom (39) reveals the C-terminal domain motions occur around three different rotation axes (see the gray arrows in Fig. 4). Apo- and holoKPR are both in an open form and are closer to monomer A than to monomer B (see Fig. 4 and Table 3). No hinge bending domain closure is identified in either apo/holo or holo/monomer A. In each case the rotation axis has a component perpendicular to the active site cleft (see Fig. 4A and B), indicating that a degree of shear-like domain motion occurs. In contrast, hinge bending is observed between the two KPR-NADP⁺-pantoate ternary complexes (Fig. 4C). The interdomain region formed by resi-
dyes 169–176 (shown in orange in Fig. 4C) acts a mechanical hinge (39). A 14° rotation around a hinge axis parallel to the active site cleft triggers the domain closure to produce the catalytic ternary complex (see supplemental Movie S1).

**Cofactor Binding**—The cofactor binds in an extended conformation at the active site cleft in both ternary complexes (Figs. 2 and 3). The non-covalent interactions with the protein residues are shown schematically in Fig. 5. The main interactions of NADP⁺ in monomer A are similar to those observed in the structure of holoKPR by Lobley et al. (18). The 2'-phosphate group makes two hydrogen bonds with the guanidinium group of Arg31, Leu6, Leu71, and Gln75; the pyrophosphate group is hydrogen-bonded to the backbone amides of Ala10 and Leu11 from the glycine-rich region; the nicotinamide-ribose 2'- and 3'-hydroxyls form hydrogen bonds with the carboxylate group of Glu92 (Fig. 6A) and with the backbone amide of Asn98; the nicotinamide group makes hydrogen-bond contacts with the side chains of Leu11 and Thr118; and the nicotinamide carboxyl and amide groups form hydrogen bonds with the backbone amide and carbonyl of Ala222.

The binding mode of NADP⁺ in monomer B is nearly identical to that in the open complex, the only difference being a subtle movement of the 2'-phosphate moiety (indicated by an arrow in Fig. 4C). However, several interactions with the protein differ substantially due to movements of active site residues during the hinge bending (see Figs. 3 and 5). First, the side chain of Arg253 swings by ~8 Å to form two hydrogen bonds with the 2'-phosphate of the cofactor, leaving only one hydrogen bond from Arg31. Second, rotation of the side chain of Asn98 brings its amide group into hydrogen-bond contact with the nicotinamide ribose 2'-hydroxyl. Third, the domain closure brings the a9-loop–a10 motif on top of the C-terminal domain toward the reaction center, allowing Asn241 to form a hydrogen bond to the nicotinamide carbonyl oxygen as well as to the C4 hydroxyl of pantoate. Finally, a subtle movement of the side chain of Lys72 allows hydrogen bonding with the pyrophosphate of NADP⁺.

**Substrate Binding**—The ternary complex crystal structures here reported provide the first evidence of substrate binding to KPR. The hinge bending brings the C2 carbon of pantoate ~3.3 Å from the C4 carbon of NADP⁺ (Fig. 4C). This distance is optimal for hydride transfer (40), suggesting monomer B is the precatalytic ternary complex. Significantly, the C2 hydroxyl of pantoate forms a hydrogen bond with the side chain amine of Lys176 at 2.7 Å of distance (Figs. 3B and 6A). This is the first direct evidence of this interaction, and of the "active state" conformation of Lys176 previously proposed by Lobley et al. (18). The side chain amine of Lys176 forms additional hydrogen bonds with the side chain carboxyl of Asn98 and main chain carboxyl of Thr118 (Fig. 6A). In monomer B, pantoate is fully enclosed between the two domains in such a way as to exclude access by the solvent. The carboxylic group forms hydrogen bonds with the side chain hydroxyl and the backbone amide of Ser244 and with the side chain amide of Asn98. The C2 hydroxyl forms an additional hydrogen bond with the side chain amide of Asn180. The C4 hydroxyl makes two hydrogen bonds with the side chain amides of Asn194 and Asn241. Finally, the C3 dimeth-
yl group sits in a hydrophobic pocket defined by the side chains of Thr\(^{119}\), Val\(^{179}\), Ile\(^{183}\), Val\(^{234}\), and Thr\(^{238}\).

In monomer A pantoate is more than 6.5 Å away from NADP\(^+\), with water molecules filling the gap left in the active site. Throughout the closed-open conformational change, the carboxylate group of pantoate remains anchored to Ser\(^{244}\) and Asn\(^{98}\), and the C2 and C4 hydroxyls maintain their hydrogen bonds with Asn\(^{180}\) and Asn\(^{194}\), respectively (Fig. 6B). The hydrogen bond to Asn\(^{180}\) appears to be important to stabilize the C2 hydroxyl of pantoate. This interaction may account for the enzyme selectivity for pantoate over ketopantoate (20-fold with apo-KPR and 80-fold with KPR-NADP\(^+\)). The K176A mutant exhibited the lowest activity among all the mutants analyzed, showing a 1670-fold decrease in \(k_{\text{cat}}\). The N98A and E256A mutations decreased \(k_{\text{cat}}\) by 10-fold. All three mutants also exhibited large decreases in \(k_{\text{cat}}/K_m\) for ketopantoate. In contrast, the other mutants showed \(k_{\text{cat}}\) values within 2-fold of the WT enzyme (Table 4). These kinetic results are consistent with previous results of in vivo studies, which showed that N98A, K176A, and E256A were unable to complement a pantoate auxotroph of Salmonella typhimurium (18).

The K176A mutation increased the \(K_d\) of the substrates by 4-fold and decreased the \(K_d\) of the coenzymes, albeit more significantly for NADPH than NADP\(^+\) (Table 5). Both N98A and E256A mutations affected the thermodynamics of coenzyme binding, leading to larger favorable \(\Delta H\) and less favorable \(-T\Delta S\) relative to WT (Table 5). Interestingly, the N98A mutation exhibited a 6-fold increase in binding affinity for both
NADPH and NADP⁺. No heat effect was observed by ITC between N98A or E256A and ketopantoate or pantoate (at concentrations up to 50 mM).

The S244A mutant showed comparable $k_{cat}$ to WT enzyme. However, the $K_m$ value for ketopantoate increased to 7 mM, resulting in a 380-fold decrease in $k_{cat}/K_m$ (Table 4). These results suggest that the S244A mutation destabilizes both the ground state and the transition state to a comparable extent. The $K_d$ for ketopantoate and pantoate to S244A increased by 20-fold relative to WT, consistent with interactions of Ser244 with the substrate carboxylate group (Fig. 6). The thermodynamic parameters for cofactor binding were not significantly changed upon mutation of Ser244.

The R31A and K72A mutants exhibited slightly higher $k_{cat}$ than WT but increased the $K_m$ for NADPH by 4 and ~10-fold, respectively (Table 4). Further evidence for the involvement of Arg31 and Lys72 in cofactor recognition came from ITC. As compared with WT enzyme, the $K_d$ for NADPH and NADP⁺ to K72A are increased 18- and 32-fold, respectively (Table 5). The R31A mutation increased the $K_d$ for NADP⁺ by 14-fold but did not significantly increase the $K_d$ for NADPH. The K72A mutant exhibited a ~60-fold increase in $K_m$ for ketopantoate. This was surprising since Lys72 is more than 7 Å away from pantoate in the crystal structure (Fig. 6). Furthermore, the calorimetric studies showed no significant changes in substrate binding to K72A as compared with WT (Table 5).

### DISCUSSION

Previous mutagenesis studies (9) and analysis of the crystal structure of the KPR-NADP⁺ complex (18) have led to the proposal that Lys176 acts as the general acid in the physiologically important direction of ketopantoate reduction by donating a proton to the developing alkoxide on ketopantoate. The crystal structure of the ternary complex KPR-NADP⁺-pantoate in its closed form provides the first evidence of a hydrogen bond between Lys176 and the C2 oxygen of product pantoate, observed at 2.7 Å distance.

A sequential ordered bi:bi kinetic mechanism (Scheme 1) for KPR activity was proposed by Zheng and Blanchard (10) based on product inhibition analyses. Sequential ordered mechanisms often occur in reactions catalyzed by dehydrogenases, with the coenzyme binding first (41). This has been explained by a conformational change upon binding of the nicotinamide adenine dinucleotide that increases the affinity of the enzyme for the second substrate (42). Our ITC results show that the binding of ketopantoate is enhanced by more than 2
orders of magnitude in the presence of NADPH, consistent with the compulsory ordered mechanism. The binding of NADPH concomitantly protects the enzyme from pantoate inhibition, presumably due to a steric repulsion between the hydrogens present in each reduced center. This selectivity for ketopantoate over pantoate is only observed with the KPR
ketopantoate complex or the apoenzyme, which are both in a more open conformation. We speculate that the KPR-NADPH complex must be in a more closed conformation than KPR-NADP+. This conclusion is consistent with (a) the differences in substrate binding cooperativity between the two homologues, (b) the different effects of R31A and K176A on coenzyme binding, and (c) the different ΔG, ΔS, and heat capacity ΔCp, changes associated with coenzyme binding (18).

Previous kinetic studies suggest that hydride transfer is not rate-limiting in catalysis (9, 10, 43). Here we show that the mutants R31A and K72A bind NADP+ less well but have an increased kcat. Conversely, the N98A mutant binds NADP+ more tightly and has a decreased kcat. These results are consistent with dissociation of NADP+ being partially rate-limiting for catalysis.

The two crystal structures of ternary complexes reported here and the previously reported structures provide snapshots of the enzyme in action (see supplemental Movie S2). These structures and additional kinetic and thermodynamic studies with active site mutants allow us to propose a detailed catalytic mechanism for E. coli ketopantoate reductase (Scheme 2). The initial binding of NADPH with recognition of its 2’-phosphate and pyrophosphate groups by the side chains of Arg71 and the conserved Lys72 induces conformational changes to provide an active site environment that favors ketopantoate binding and discriminates against pantoate binding. An extended hydrogen bonding network provided by conserved residues Glu256 and Asn98 orients the ribose-nicotinamide moiety of the cofactor in the conformation required for productive substrate binding and optimal hydride transfer. A significant hinge bending encloses the active site around ketopantoate to provide a solvent-inaccessible environment in which catalysis occurs. After binding of ketopantoate, the C4 pro-S hydride of NADPH is transferred to the si face of ketopantoate, with protonation of the developing alkoxide by Lys176 in its active state (Scheme 2, left). The substrate is locked during each step of the reaction via hydrogen bonds of its carboxylate group to the conserved Ser244. Other binding interactions are provided by the side chains of four asparagine residues, Asn98, Asn180, Asn194, and Asn241. In addition to binding the substrate, Asn98 plays a central role in the catalytic mechanism by stabilizing the active conformation of Lys176 and by promoting the dissociation of NADP+. The opening of the hinge subsequently allows the reprototation of Lys176 from solvent and its return to the resting state (Scheme 2, right). The loss of hydrogen bonds from Lys176 ends the catalytic cycle.

Conclusions—Ketopantoate reductase catalyzes an essential step in the biosynthesis of pantothenate. This paper reports the crystal structure of the enzyme captured in a precatalytic ter-

### Table 5: Thermodynamic parameters for cofactor and substrate binding with WT and mutant forms of KPR

| KPR    | n | Kd (μm) | ΔH (kcal/mol) | ΔS (kcal/mol) |
|--------|---|---------|---------------|---------------|
| WT     | 1.0 | 0.4 ± 0.2 | -4.5 ± 0.6 | -4.3 ± 0.8 |
| R31A   | 1.1 | 0.7 ± 0.3 | -3.8 ± 0.5 | -4.7 ± 0.7 |
| K72A   | 1.0 | 0.7 ± 0.3 | -4.5 ± 0.6 | -4.3 ± 0.8 |
| N98A   | 1.1 | 0.07 ± 0.01 | -2.3 ± 0.4 | -7.5 ± 0.3 |
| K176A  | 1.1 | 0.9 ± 0.1 | -5.0 ± 0.4 | -3.5 ± 0.5 |
| S244A  | 0.9 | 0.18 ± 0.08 | -11.3 ± 0.4 | 2.6 ± 0.8 |
| E256A  | 0.5 | 0.6 ± 0.3 | -11.3 ± 0.4 | 2.6 ± 0.8 |

All ITC titrations were run at 27 °C in 50 mM HEPES-HCl, pH 7.6. Errors quoted are S.D. of the mean parameter values from at least duplicate titrations. Otherwise, errors are those returned by Origin on the curve fitting, not determined.

### Table 6: Structure of KPR Ternary Complexes

| Structure   | Axis A Coordination | Axis B Coordination |
|-------------|---------------------|---------------------|
| WT          | (0,0,0)             | (0,0,0)             |
| R31A        | (0,0,0)             | (0,0,0)             |
| K72A        | (0,0,0)             | (0,0,0)             |
| N98A        | (0,0,0)             | (0,0,0)             |
| K176A       | (0,0,0)             | (0,0,0)             |
| S244A       | (0,0,0)             | (0,0,0)             |
| E256A       | (0,0,0)             | (0,0,0)             |

with active site mutants allow us to propose a detailed catalytic mechanism for E. coli ketopantoate reductase (Scheme 2). The initial binding of NADPH with recognition of its 2’-phosphate and pyrophosphate groups by the side chains of Arg71 and the conserved Lys72 induces conformational changes to provide an active site environment that favors ketopantoate binding and discriminates against pantoate binding. An extended hydrogen bonding network provided by conserved residues Glu256 and Asn98 orients the ribose-nicotinamide moiety of the cofactor in the conformation required for productive substrate binding and optimal hydride transfer. A significant hinge bending encloses the active site around ketopantoate to provide a solvent-inaccessible environment in which catalysis occurs. After binding of ketopantoate, the C4 pro-S hydride of NADPH is transferred to the si face of ketopantoate, with protonation of the developing alkoxide by Lys176 in its active state (Scheme 2, left). The substrate is locked during each step of the reaction via hydrogen bonds of its carboxylate group to the conserved Ser244. Other binding interactions are provided by the side chains of four asparagine residues, Asn98, Asn180, Asn194, and Asn241. In addition to binding the substrate, Asn98 plays a central role in the catalytic mechanism by stabilizing the active conformation of Lys176 and by promoting the dissociation of NADP+. The opening of the hinge subsequently allows the reprototation of Lys176 from solvent and its return to the resting state (Scheme 2, right). The loss of hydrogen bonds from Lys176 ends the catalytic cycle.

Conclusions—Ketopantoate reductase catalyzes an essential step in the biosynthesis of pantothenate. This paper reports the crystal structure of the enzyme captured in a precatalytic ter
nary complex with NADP$^+$ and pantoate bound, giving the first direct evidence of a hinge bending domain closure in KPR and of a hydrogen bond between Lys$^{176}$ and the substrate. The structure and thermodynamics of ternary complexes provide insights into the interplay of conformational changes and cooperativity in the sequential ordered mechanism. Additional kinetic and calorimetric studies with site-directed mutants have elucidated the roles of active site residues Arg$^{31}$, Lys$^{72}$, Asn$^{98}$, Lys$^{176}$, Ser$^{244}$, and Glu$^{256}$ in substrate recognition and catalysis. Our crystal structure has highlighted the importance of other residues, including Asn$^{180}$, Asn$^{194}$, Asn$^{241}$, and Arg$^{253}$, which will be the subject of future mutagenesis studies. Finally, the crystal structure of the closed ternary complex will be a useful template for designing inhibitors that mimic the structure of the transition state during hydride transfer. The relevance of ligand-induced domain movements in drug design has been recently stressed (44) and may prove useful in designing inhibitors against KPR.

Acknowledgment—We thank Sarah L. Maslen for assistance with mass spectrometry of the KPR mutants.

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