

1 Catalytic Mechanism and Allosteric Regulation of UDP-Glucose 2 Pyrophosphorylase from *Leishmania major*

3 Jana Fühling,[†] Johannes T. Cramer,[†] Françoise H. Routier,[†] Anne-Christin Lamerz,^{†,‡} Petra Baruch,[‡]
4 Rita Gerardy-Schahn,[†] and Roman Fedorov^{‡,§,*}

5 [†] Institute for Cellular Chemistry, OE4330, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

6 [‡] Research Division for Structural Analysis, OE8830, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

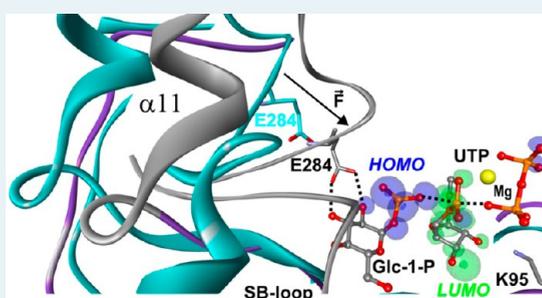
7 [§] Institute for Biophysical Chemistry, OE4350, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

8 **S** Supporting Information

9 **ABSTRACT:** UDP-glucose pyrophosphorylase (UGP) is a nucleotidyl-
10 transferase of central importance in all organisms and considered an
11 attractive drug target in the human pathogens *Leishmania* and
12 *Trypanosoma*. Here, we used wild-type and mutant *Leishmania major*
13 UGP to solve the crystal structures of postreactive, UTP, and UDP-Glc
14 bound states and performed kinetic and theoretical chemistry analysis of
15 the enzymatic reaction. The new data filled critical gaps in the
16 knowledge of the UGP mechanism and allowed reconstructing the
17 complete enzymatic cycle on three levels: global (movements of
18 molecular functional blocks), local (behavior of separate residues), and
19 chemical (quantum mechanical description of enzymatic reaction).

20 Results were integrated into a model of UGP activity describing structural changes along the cycle, the mechanisms of substrate
21 binding, UGP catalysis, and product release. Our study revealed the mechanisms of allosteric regulation common for
22 nucleotidyltransferases and, in particular, the mechanical control of the chemical reaction in the active site.

23 **KEYWORDS:** nucleotidyltransferase, catalysis, allosteric regulation, molecular mechanism, *Leishmania*, *Trypanosoma*,
24 complete enzymatic cycle, mechanochemistry



25 ■ INTRODUCTION

26 Enzymes of the large superfamily of nucleotidyltransferases
27 (nucleotide triphosphate (NTP) transferases) catalyze key
28 cellular processes in all kingdoms of life.¹ Within the wide
29 spectrum of functions, members of this superfamily are
30 responsible for DNA repair, RNA editing, and activation of
31 metabolites used in catabolic pathways, as well as detoxification
32 and antibiotic resistance mechanisms.¹ Despite large diversity
33 existing at primary and quaternary structure levels (mono-, di-,
34 tetra-, hexa-, and octameric forms have been described^{2–6}),
35 nucleotidyltransferases commonly adopt Rossmann-like $\alpha/\beta/\alpha$
36 sandwich folds in their catalytic domains,⁶ require Mg^{2+} for
37 catalytic activity,^{7–9} and follow a sequentially ordered bibi
38 catalytic mechanism, with the NTP binding first.^{4,5,10,11} In
39 keeping with their central cellular positions, nucleotidyltrans-
40 ferases have been demonstrated to be a subject of elaborate
41 regulatory mechanisms,¹ including allosteric effectors, redox
42 regulation, phosphorylation^{12–15} and sequestration of active
43 enzymes by oligomerization.^{3,16} Although intensively studied at
44 both the kinetic and structural levels,^{4,5,10,11} molecular details
45 explaining common functional properties of nucleotidyltrans-
46 ferases, such as the bibi kinetics and the role of the essential
47 Mg^{2+} ion, are either missing or equivocally discussed.^{7,10}
48 Similarly, it remains unknown how the various levels of
49 structural organization combine with allosteric regulation to

50 affect the reaction cycles of these enzymes. Elucidating these
51 mechanisms is, however, essential for the fundamental
52 understanding of nucleotidyltransferases and the development
53 of new concepts to specifically inhibit individual members.

54 Recently, we cloned UDP-glucose (UDP-Glc) pyrophos-
55 phorylase from *Leishmania major* (LmUGP).² This enzyme
56 catalyzes the reversible conversion of glucose-1-phosphate
57 (Glc-1-P) and uridine 5'-triphosphate (UTP) to UDP-Glc
58 and inorganic pyrophosphate (PP_i) in the presence of Mg^{2+}
59 ions. Very different from all known pro- and eukaryotic UGPs,
60 the LmUGP is an active monomer with no capability to form
61 higher oligomers that, for example, in plants represent
62 sequestered enzyme forms.^{3,16} This unique feature together
63 with the fact that UGP is a promising target for the
64 development of specific antiparasitic agents^{17,18} motivated us
65 to investigate the function of this enzyme at the macro-
66 molecular, atomic, and chemical levels.

67 Previously obtained open and closed structures of LmUGP
68 (pdb codes: 2OEF and 2OEG, respectively) revealed major
69 conformational change between these two states.¹⁹ To
70 understand these molecular movements and reconstruct a

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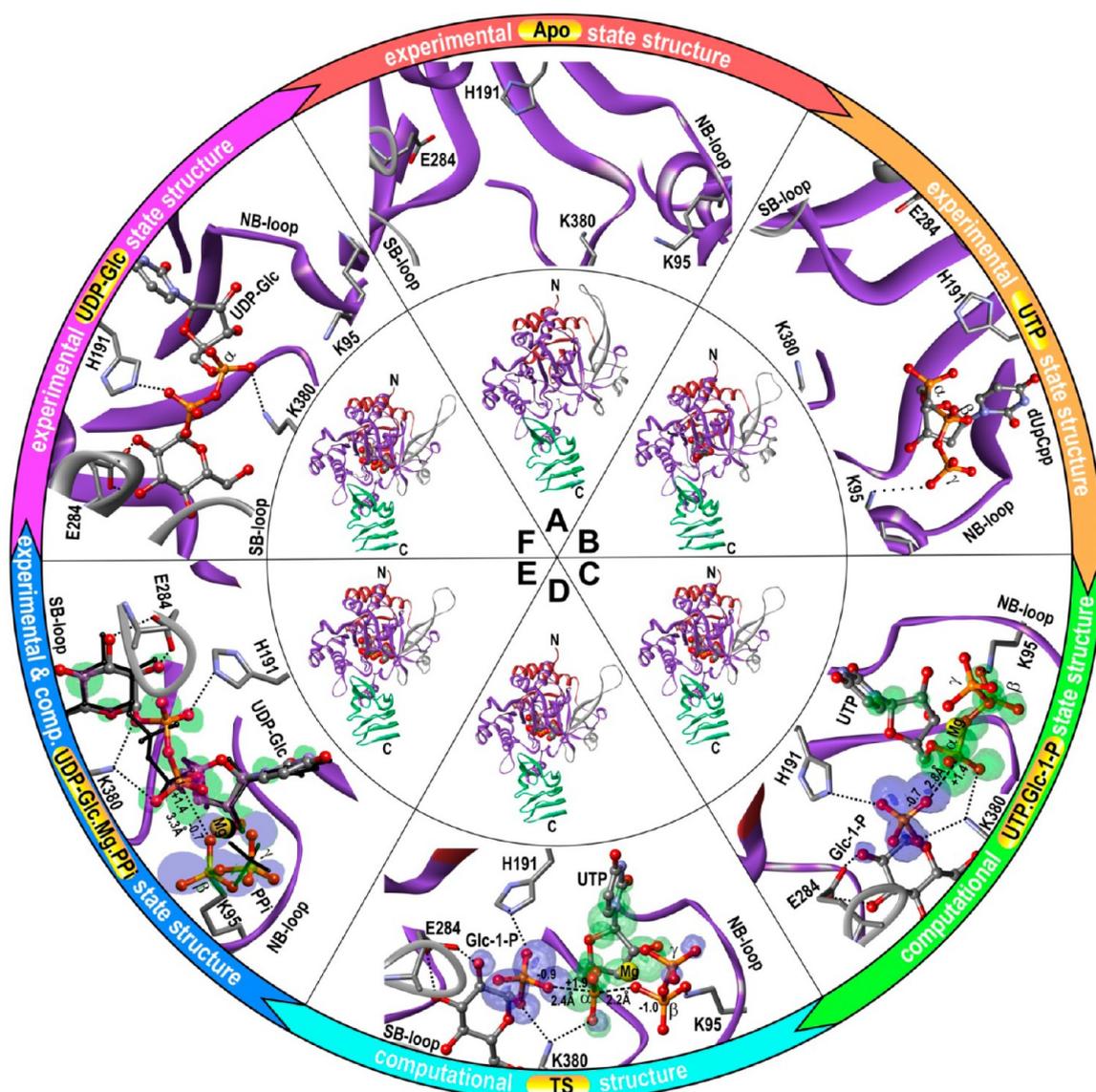


Figure 1. Enzymatic cycle of LmUGP. The inner circle shows the overall views of LmUGP for each state in ribbon representation. The outer circle shows the close-ups of the UGP active site. The N-terminal, C-terminal, and central catalytic domains are shown in red, green, and blue, respectively. The lock region is shown in gray. The substrates are shown in a Corey–Pauling–Koltun representation. The outer segments C, D, and E contain graphical representations of HOMO (blue) and LUMO (green). Only lobes of the same sign localized on reacting atoms are shown. (A) Open state (apo form) representations based on the experimental structure 2OEF.¹⁹ The N-terminal/catalytic and C-terminal domains are at their maximum distance, the seven-stranded β -sheet is relaxed, the lock is open. Only the binding site for UTP is present. (B) Crystal structure of LmUGP-dUpCcp complex representing the UTP state. The seven-stranded β -sheet and the NB loop acquire intermediate conformations between the open and the closed states, and the lock region remains open. The phosphates are located in the interface between the catalytic and C-terminal domains with the γ -phosphate stabilizing the NB loop. The binding pocket for the glucose ring is formed. (C) QM/MM model of the prereactive state. The whole structure is in the closed state; both substrates are stabilized in position for the reaction. (D) The trigonal bipyramidal geometry of the transition state. (E) QM/MM (colored) and experimental (black) models of the postreactive state. (F) Experimental structure of UDP-Glc state (2OEG).¹⁹ The pyrophosphate dissociation leads to the relaxation of the NB loop and the activation of the lock mechanism for opening the LmUGP structure.

71 comprehensive picture of the enzymatic cycle, we aimed to
 72 obtain additional intermediate state geometries. Here, we
 73 determined the crystal structures of wild-type and mutant
 74 LmUGP in the presence of substrates, products, or their
 75 analogs.

76 The new structures for the first time revealed the binding
 77 sites for all UGP products, elucidated the key role of the UTP
 78 nucleoside and γ -phosphate in UGP activation, and helped
 79 explain the allosteric mechanisms that accompany binding and
 80 stabilization of both substrates of the bi-reacting LmUGP.
 81 Moreover, novel insights into the elements responsible for

substrate binding and product release have been obtained. Our
 82 structural analysis is supported by original kinetics data
 83 presented in this study and by published data for the forward
 84 and backward reactions of the wild-type LmUGP and its
 85 functional mutants.^{2,19} The ab initio quantum mechanics/
 86 molecular mechanics (QM/MM) calculations of the LmUGP
 87 reaction pathway helped elucidate electronic effects and the
 88 roles of Mg^{2+} and active site residues in UGP catalysis.
 89

The resulting model of UGP activity describes structural
 90 changes along the catalytic cycle, the mechanisms of substrate
 91 binding, UGP catalysis, and product release (Figure 1). With 92 ft

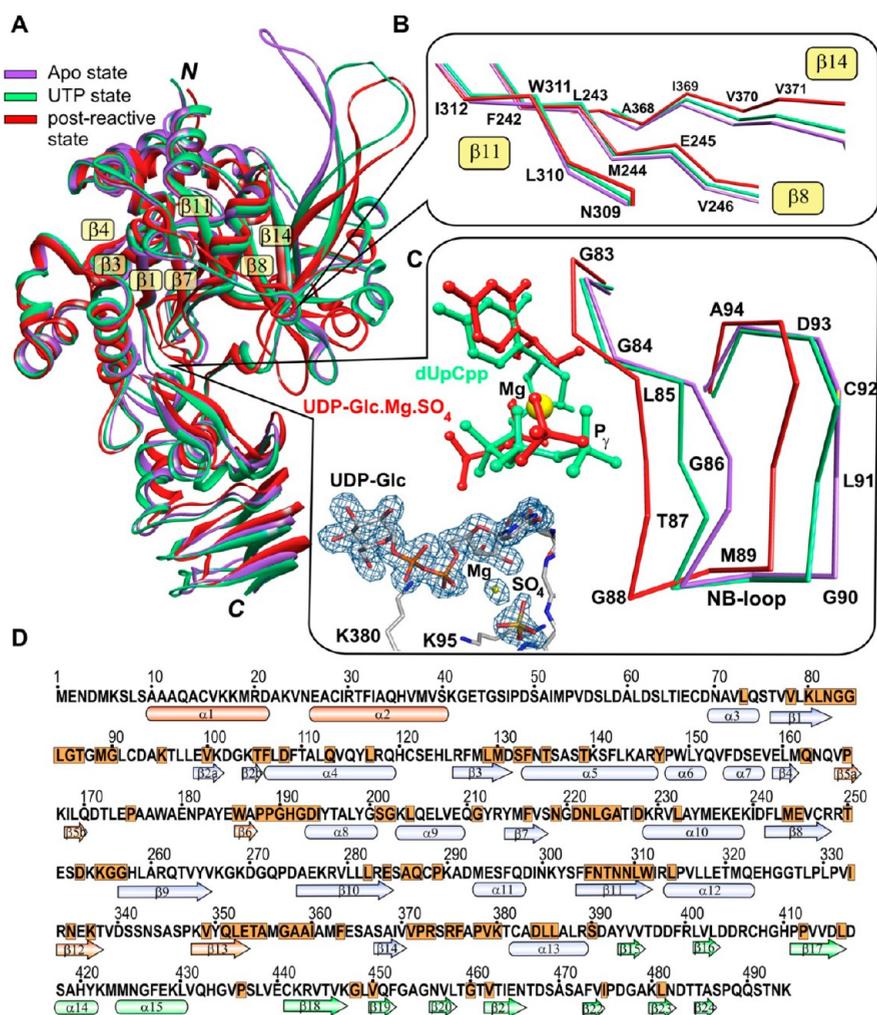


Figure 2. (A) Superposition of the LmUGP structures in the apo state (blue; PDB code: 2OEF¹⁹), the UTP state (green; LmUGP-dUpCpp complex), and the postreactive state (red; LmUGP-UDP-Glc-Mg²⁺-SO₄ complex) illustrates the overall conformational changes upon binding the first and second substrates. The torsional deformation of the seven-stranded β -sheet in the area near the glucose binding site is shown in panel B. Panel C shows structural changes in the NB loop area and the $F_{\text{obs}} - F_{\text{calc}}$ electron density omit map contoured at 3.0σ around the ligands UDP-Glc, Mg²⁺ and SO₄²⁻ in the wild-type LmUGP postreactive state structure. (D) Amino acid sequence of LmUGP. The secondary structure elements of the N-terminal domain are red; catalytic domain, blue; and C-terminal domain, green.¹⁹ The residues conserved in UGPs from different organisms are highlighted in orange.

93 this description of the complete reaction cycle of LmUGP, we
 94 provide novel insights into the function of nucleotidyltrans-
 95 ferases and a general guide for the in-depth mechanistic analysis
 96 of these enzymes. Finally, we demonstrate how allosteric
 97 mechanisms control specific recognition of glucose by LmUGP
 98 and regulate the chemical reaction in the active site via
 99 mechanical force transduction.

100 ■ EXPERIMENTAL SECTION

101 **Protein Preparation and Crystallization.** Untagged and
 102 C-terminally His₆-tagged wild-type *L. major* UGP were
 103 prepared as described by Lamerz et al.² Mutant proteins
 104 L281D and H191L were prepared as described by Steiner et
 105 al.¹⁹ The E284A mutant was created by overlap extension PCR
 106 using mutagenesis primers with the sequences
 107 gctgaggGCGtcgcg (sense) and gcggaCGCccgcgacg (antisense),
 108 respectively, in which the mutated codon is given in uppercase.
 109 The C-terminally His₆-tagged E284A mutant protein was then
 110 expressed and purified in the same way as the His₆-tagged wild-
 111 type protein.² Crystals of the wild-type enzyme complexes were

grown at 20 °C by vapor diffusion in hanging drop geometry 112
 with 500 μ L of reservoir solution. Crystals of the L281D and 113
 the H191L mutants were grown at 4 and 20 °C, respectively, in 114
 sitting drop geometry with 500 μ L of reservoir solution. For the 115
 UTP state, 1 mM dUpCpp and 4 mM MgCl₂ were added to the 116
 wild-type protein (12.4 mg/mL; 10 mM Tris-HCl, pH 7.5; 117
 100 mM NaCl; 2 mM MgCl₂; 2 mM DTT). A 1.0 μ L portion 118
 of the resulting complex was mixed 1:1 with the reservoir 119
 solution (0.1 M MES, pH 6.4; 20% w/v PEG-4000; 200 mM 120
 Li₂SO₄). For the postreactive state, 1.1 μ L of the wild-type 121
 protein (7 mg/mL; 10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 122
 2 mM MgCl₂; 2 mM DTT; 4 mM UDP-Glc) was mixed 1:1 123
 with the reservoir solution (0.1 M Bis-Tris, pH 6.0; 22% w/v 124
 PEG-MME-2000; 20 mM (NH₄)₂SO₄). The L281D mutant 125
 was concentrated to 2.3 mg/mL in the same buffer adjusted to 126
 pH 7.8. The reservoir solution contained 0.1 M Bis-Tris, pH 127
 6.6, and 28% w/v PEG-MME-2000. In the case of the H191L 128
 mutant, the protein buffer contained 10 mg/mL LmUGP_{H191L}; 129
 10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM MgCl₂; 2 130
 mM DTT; 4 mM UTP; and 4 mM Glc-1-P. The reservoir 131

132 solution contained 10% v/v glycerol; 0.05 M citrate buffer, pH
133 5.2; and 1.1 M sodium citrate. Prior to flash-cooling, all crystals
134 were rinsed in reservoir solution supplemented with 20%
135 ethylene glycol for cryoprotection.

136 **In Vitro Activity Measurements and Kinetic Data**
137 **Analysis.** To calculate reaction energy parameters, the in vitro
138 activity of wild-type LmUGP was measured at 25 and 37 °C
139 using the untagged recombinant protein. In the forward
140 direction of the reaction (production of UDP-Glc), formation
141 of pyrophosphate was continuously detected using the
142 EnzChek Pyrophosphate Assay Kit (Molecular Probes) in a
143 buffer containing 50 mM Tris/HCl, pH 7.8, and 10 mM
144 MgCl₂. UTP was kept at a saturating concentration of 1 mM,
145 and the concentration of Glc-1-P was varied between 0.1 and 3
146 mM. The backward reaction (formation of UTP) was
147 monitored using the continuous assay described by Damerow
148 et al.²⁰ Here, UDP-Glc was supplied at a saturating
149 concentration of 1 mM, and pyrophosphate concentration
150 was varied between 0.1 and 2 mM. For the forward and
151 backward reaction activity assays, a 1.84 mg/mL UGP stock
152 was diluted 1:40 000 and 1:10 000, respectively. The reaction
153 was started by addition of 10 μL of diluted UGP unto a final
154 reaction volume of 100 μL. The resulting values of [E]₀ for the
155 forward and backward reactions were 8.6915 × 10⁻¹¹ M and
156 3.4766 × 10⁻¹⁰ M, respectively. Product formation was
157 continuously monitored at 360 nm in a microplate reader
158 (Power-Wave 340 KC4 system, Bio-Tek). The rates of product
159 release were plotted against the varied substrate concentration,
160 and K_m and V_{max} were calculated using the nonlinear fitting
161 function in PRISM software (GraphPad Software, Inc.). The
162 free-energy barriers resulting from the experimental V_{max} values
163 (Supporting Information Figure S2A,B) were calculated
164 assuming that the reaction catalyzed by LmUGP proceeds via
165 the sequentially ordered bi-bi catalytic mechanism common for
166 nucleotidyltransferases, with a single barrier between the pre-
167 and postreactive states. In this case, the rate-limiting step
168 constant corresponding to the substrate-to-product conversion
169 is identical to $k_{\text{cat}} = V_{\text{max}}/[E]_0$, and the reaction free energy
170 barrier can be estimated according to the transition state theory
171 (TST) as $\Delta G^\ddagger = -RT \ln(k_{\text{cat}}h)/(k_{\text{B}}T)$, where R , k_{B} , and h are
172 the ideal gas, Boltzmann's, and Planck's constants, respectively,
173 and T is the absolute temperature.

174 To compare kinetic parameters of the LmUGP E284A
175 mutant and wild-type LmUGP (both C-terminally His-tagged),
176 activity measurements at 25 °C in the forward direction of the
177 reaction were carried out and evaluated as described above,
178 with the following modifications: UTP concentration was
179 varied between 0.05 and 1 mM at a saturating supply of 2 mM
180 Glc-1P, and titration of Glc-1P between 0.05 and 2 mM was
181 performed at a saturating concentration of 1 mM UTP.

182 **Diffraction Data Collection and Structure Determi-**
183 **nation.** Diffraction data were collected at the EMBL beamlines
184 X11 and X13 at the DORIS storage ring, DESY, Hamburg, and
185 the beamlines ID23-2 and ID29, at ESRF, Grenoble, using
186 MAR CCD and Dectris Pilatus 6 M detectors (see Supporting
187 Information Table S1 for details), and reduced with the XDS
188 program package.²¹ The structures of LmUGP_{wt}·dUpCp
189 complex and LmUGP_{H191L} mutant was solved by molecular
190 replacement (CCP4 software suite^{22,23}) using the structure of
191 LmUGP apo form (pdb code: 2OEF¹⁹) as an initial model.
192 Structure determination of LmUGP_{wt}·UDP-Glc-Mg²⁺·SO₄ and
193 LmUGP_{L281D}·UDP-Glc complexes was initiated by a round of
194 CNS^{24,25} rigid body refinement using the protein part of the

LmUGP coordinates for the closed conformation of the 195
enzyme (pdb code: 2OEG¹⁹) as an initial model. Refinement 196
was continued with CNS, SHELX,²⁶ and REFMAC²⁷ and 197
included simulated annealing and individual B-factor refine- 198
ment. The initial electron density maps were calculated using 199
ARP/wARP.²⁸ During cyclic rounds of refinement and manual 200
rebuilding using the COOT²⁹ program, ligands and solvent 201
molecules were included in the models (see Figures 2C and 202
Supporting Information S1A–D). The final models displayed 203
good stereochemistry (see Supporting Information Table S1). 204
Structure comparisons were performed using SSM and LSQ 205
superpose tools of COOT. 206

Molecular Dynamics Simulations. To study the effect of 207
the E284–glucose interaction on the conformation of LmUGP, 208
molecular dynamics (MD) simulations were performed using 209
the CNS^{24,25} program package. The starting geometry was 210
obtained from the coordinates of the LmUGP_{wt}·dUpCp 211
complex (UTP state) and the glucose coordinates from the 212
superimposed structure of the LmUGP_{wt}·UDP-Glc-Mg²⁺·SO₄ 213
complex (postreactive state). In this geometry, the distances 214
between the oxygen atoms of the E284 carboxyl group and the 215
2',3'-oxygen atoms of glucose were close to 6 Å. At the start of 216
the simulations, the hydrogen bond distance restraints between 217
these atoms were assigned. The Cartesian MD simulations were 218
performed for 1 ns MD trajectory at a constant temperature of 219
300 K. At the end of the dynamic trajectory, the model 220
geometry was optimized using CNS-DEN refinement³⁰ without 221
experimental energy terms. 222

Quantum Chemical Modeling of LmUGP Reaction 223
Pathway. The QM/MM simulations of the LmUGP reaction 224
pathway were performed using the Firefly Quantum Chemistry 225
package,³¹ based in part on the GAMESS (US)³² source code. 226
The QM subsystem included the substrates, water, Mg²⁺, and 227
fractions of the side chains for the protonated residues K95, 228
H191, and K380. It contained 94 atoms in total and had a 229
charge of -1. The rest of the protein was included into the MM 230
part. Geometry optimizations, Hessian calculations, saddle 231
point location, and intrinsic reaction coordinate (IRC) 232
simulations in the QM part were carried out using density 233
functional theory (DFT) with the B3LYP hybrid function- 234
tional,^{33,34} Vosko–Wilke–Nusair electron gas formula 5 235
correlation,^{35,36} and the valence double- ζ polarized basis set 236
6-31G* for all atoms. The MM3 force field parameters^{37–39} 237
were used for the MM part. The starting geometry for the 238
saddle point location search was generated from the 239
coordinates of the postreactive state complex and the substrate 240
coordinates from the structure of LmUGP·dUpCp complex 241
obtained in this work. This structure was optimized using an 242
unconstrained QM/MM geometry minimization procedure. 243

The search for the saddle point location was started by 244
successively decreasing the distance between the reacting atoms 245
O_{3P}^{Glc-1-P} and P _{α} ^{UTP} in the direction of the forward reaction and 246
performing constrained geometry minimization at each step. 247
Upon reaching the flat conformation of the P _{α} O₃ group of 248
UTP, the precise location search of the saddle point was 249
performed using a quadratic approximation augmented Hessian 250
technique.^{40–42} The transition state geometry was located at a 251
point with a single imaginary frequency of Hessian. After 252
finding the transition state, the structures of the pre- and 253
postreactive states were obtained by descent forward and 254
backward from the saddle point along the steepest descent path 255
in mass-weighted Cartesian coordinates using the IRC 256
method.⁴³ The resulting models of pre- and postreactive states 257

258 corresponded to the true minima on the potential energy
 259 surface with all real vibrational frequencies. These structures
 260 were used for further analysis and comparison with
 261 experimental data. The free energy barriers for forward and
 262 backward reactions were calculated using harmonic normal
 263 mode approximation.

264 ■ RESULTS

265 **LmUGP·dUpCpp Complex and the Mechanism of UTP**
 266 **Binding.** To study the effect of UTP-binding on the structure
 267 and function of LmUGP, we crystallized LmUGP in the
 268 presence of the nonreactive UTP analog dUpCpp (see
 269 Experimental Section for details). The structure solved by
 270 molecular replacement and refined to 3.0 Å resolution showed
 271 good electron density for the UTP analog bound to the active
 272 site (Supporting Information Figure S1C). The overall
 273 conformation of the enzyme is more similar to the open¹⁹
 274 than to the closed (postreactive; see below) state, with several
 275 regions acquiring intermediate conformations (Figure 2A).
 276 Compared with the open state, the large magnitude change is
 277 located in the nucleotide-binding (NB) loop, which shifts 2.4 Å
 278 toward the phosphate moiety, bounding the nucleotide pocket
 279 (Figure 2C). The seven-stranded β -sheet (β 1, β 3, β 4, β 7, β 8,
 280 β 11, β 14; Figure 2D) undergoes a torsional deformation, where
 281 strands β 1, β 7, β 8 (and adjacent region of the helix α 10), β 11,
 282 and β 14 shift toward their positions in the closed state (Figure
 283 2B), while the conformation of β 3 and β 4 remains close to the
 284 open state. The nucleoside is coordinated by residues L81,
 285 G83, G84 (NB loop), M130 (β 3), Q162 (end of β 4), G190–
 286 H191 (loop β 6– α 8), and N219–D221 (end of β 7). Most of
 287 these residues remain structurally conserved in the open, UTP,
 288 and closed states of the enzyme, which explains why UTP can
 289 bind to the apo form and activate the conformational transition.
 290 To understand the role of nucleoside binding in LmUGP
 291 activation, we crystallized the inactive LmUGP mutant
 292 H191L¹⁹ in the presence of both UTP and Glc-1-P, but no
 293 electron density was observed for the substrates. Instead, the
 294 mutant structure revealed that the side chain of L191 occupied
 295 part of the space necessary for the nucleoside binding (Figure
 296 3C). As a result, UTP could not bind, and the mutant
 297 conformation remained very close to the open form of the wild-
 298 type protein. This finding supported the primary role of the
 299 nucleoside binding in UGP activation.

300 In the X-ray structure of the LmUGP·dUpCpp complex, the
 301 phosphates of the UTP analog were coordinated by the
 302 residues in the interface between the catalytic and C-terminal
 303 domains. It is interesting that in this state, the strictly conserved
 304 residue K380, known to interact with the product UDP-Glc and
 305 to be essential for enzymatic activity,¹⁹ is located 8 Å away from
 306 the α -phosphate and does not make any interaction with the
 307 phosphate moiety (Figure 1B). The γ -phosphate forms three
 308 hydrogen bonds and several van der Waals interactions with the
 309 NB loop residues G84–T87 and a salt bridge with the strictly
 310 conserved K95. These interactions explain the large shift of the
 311 NB loop upon UTP binding. As the NB loop, in turn, is bound
 312 to the C-terminal domain via a hydrogen bond and extensive
 313 hydrophobic contacts, the new structural data elucidate the role
 314 of the γ -phosphate in the mechanism of UGP activation.

315 **Glucose-1-phosphate Binding to LmUGP.** As typical in
 316 enzymes with sequentially ordered bi-bi kinetics, the binding site
 317 for Glc-1-P does not exist in the apo form of UGP. The
 318 residues N219 (β 7), N306–N308 (β 11), and F376 (link
 319 β 14– α 13), identified as responsible for glucose binding in the

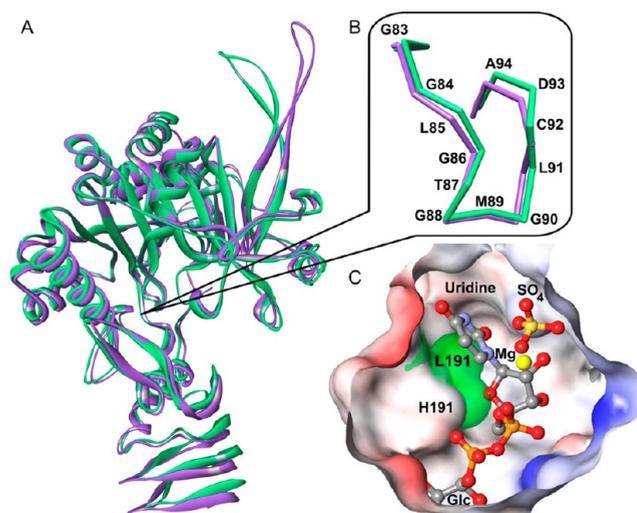


Figure 3. Structural effects of H191L mutation. Superposition of the wild-type LmUGP structure in the apo state (pdb code: 2OEF;¹⁹ green) and LmUGP_{H191L} mutant crystallized in the presence of both substrates (purple) shown for the whole enzyme (A) and for the NB loop area (B). (C) Superposition of the substrate binding pocket in the postreactive state of LmUGP_{wt} and in the LmUGP_{H191L} mutant structure. The color scheme of the wild-type protein surface corresponds to the atom charge (blue, positive; red, negative; white, neutral). The van der Waals surface of L191 from the superimposed LmUGP_{H191L} mutant structure is shown in green.

320 closed UGP structure,¹⁹ overlap with the atoms of the glucose
 321 ring in both our postreactive state and UDP-Glc¹⁹ complexes.
 322 In the UTP state, these residues are shifted deeper into the
 323 catalytic domain as a result of the torsional deformation of the
 324 seven-stranded β -sheet, which removes steric hindrances and
 325 creates a complementary surface for the glucose ring. This,
 326 together with the fact that Glc-1-P binding cannot occur
 327 without UTP binding first, as demonstrated by STD-NMR
 328 studies,² implies that the binding of the glucose ring must play a
 329 primary role in the binding of Glc-1-P. Thus, the UTP-induced
 330 deformation of the β -sheet is an important determinant of the
 331 Glc-1-P binding site formation.

332 Other structural elements known to contribute to the glucose
 333 binding in the above-mentioned closed UGP structures include
 334 the highly conserved sugar-binding (SB) loop and the strictly
 335 conserved residue E284 located at the end of β 10 (Figure 2D).
 336 These residues belong to a larger region R249–Y302
 337 comprising the SB loop, the adjoining β -hairpin β 9– β 10
 338 (handle) and helix α 11. Superposition of LmUGP structures
 339 showed that this region changes dramatically between the open
 340 and closed states (Figure 2A). Surprisingly, in the UTP-state
 341 structure, the conformation of this region remains almost
 342 identical to the open state, with the exception of the top part of
 343 the handle, which is known to be flexible, as indicated by the
 344 experimental electron density.

345 In the UTP state, both the SB loop and E284 are distanced
 346 from the glucose binding site, but E284 is located 2.0 Å closer
 347 than the nearest SB loop residue and, therefore, can bind first.
 348 To study the structural effect of the interaction between E284
 349 and the glucose ring, we have performed molecular dynamics
 350 simulations (see Experimental Section for details). As a starting
 351 model, we used the structure of the LmUGP_{wt}·dUpCpp
 352 complex (UTP state) and the glucose coordinates from the
 353 superimposed structure of the LmUGP_{wt}·UDP-Glc-Mg²⁺·SO₄
 354 complex (closed postreactive state, see below). During these 354

355 simulations, the force was applied to the side chain of E284 to
 356 bring it to its position in the closed state of the enzyme. E284 is
 357 linked to the SB loop via the β -hairpin structure of the handle
 358 and our molecular dynamics simulations suggested that pulling
 359 E284 in the direction of the glucose-binding site results in the
 360 movement of the whole region R249–Y302 toward its
 361 conformation in the closed state.

362 To test this hypothesis, we first examined the role of E284 in
 363 glucose binding. Therefore, mutant E284A was constructed and
 364 kinetically characterized in comparison with the wild-type
 365 enzyme (Supporting Information Figure S2C,D). The specific
 366 activity of the mutant E284A was reduced to less than 0.05% of
 367 the wild-type. By measuring K_m values, the affinity of the E284A
 368 mutant for UTP was found to be nearly unchanged (E284A,
 369 $73.9 \pm 5.8 \mu\text{M}$ versus wt, $101.2 \pm 7.6 \mu\text{M}$), whereas the affinity
 370 for Glc-1-P was drastically impaired (E284A, $977.4 \pm 99.5 \mu\text{M}$
 371 versus wt, $152.8 \pm 14.3 \mu\text{M}$).

372 To study the interaction between E284 and the SB loop, we
 373 investigated structural effects of the L281D mutation located in
 374 the link connecting these two areas, at the end of the strand
 375 β 10. L281 forms a part of the hydrophobic core stabilizing the
 376 base of the handle and its contact with the N-terminal and
 377 catalytic domains. The L281D mutation leads to a drop of
 378 specific activity (measured in the forward reaction) to 16.3% of
 379 the wild type.¹⁹ Because the residue is spatially remote from
 380 active site and SB loop, the mechanism of this inactivation was
 381 previously unclear. The crystal structure of the L281D mutant
 382 in complex with UDP-Glc solved in this study now shows that
 383 the mutation leads to a disruption of the β -structure of the
 384 handle (Figure 4A) and an increase in its flexibility, reflected in
 385 the weakening of experimental electron density for this region.
 386 In the mutant structure, the interactions between E284 and
 387 glucose are not changed, but the distances between E284 and
 388 the SB loop or α 11 are increased, and a lack of closure is
 389 observed in both the SB loop and the α 11 area (Figure 4B).
 390 Thus, the disruption of the β -structure caused by the L281D
 391 mutation leads to the loss of stiffness in the handle and,
 392 therefore, a reduction of its efficiency in transducing tensile
 393 force to the SB loop and α 11. This, in its turn, leads to a lack of
 394 closure in these areas (Figure 4B) and a decrease in the specific
 395 activity of the L281D mutant to 16.3% of the wild-type
 396 enzyme.¹⁹ The top of the handle features significant conformational
 397 flexibility observed in the crystal structures of LmUGP,
 398 and its length varies between species. This, in conjunction with
 399 the observed effect of the L281D mutation, implies that the
 400 residues involved in transduction of the tensile force are located
 401 at the base of the handle.

402 Comparison of the UTP state and closed postreactive state
 403 structures showed that closing the region R249–Y302 brings
 404 the strictly conserved residues H191 and K380 in a favorable
 405 position for binding Glc-1-P phosphate and leads to adjust-
 406 ments in the glucose binding site that further increase its
 407 complementarity. After closing, the region R249–Y302 is
 408 additionally stabilized by a hydrogen bond between the SB loop
 409 E251 and the C-terminal domain R443 observed in the closed
 410 state structures.

411 **Product Formation and Allosteric Regulation of**
 412 **Product Release.** By cocrystallizing LmUGP with UDP-Glc,
 413 Mg^{2+} , and SO_4^{2-} ions, the postreactive state of the enzyme
 414 could be trapped, and the structure, resolved to the high
 415 resolution of 1.6 Å. The postreactive state is the most compact
 416 of all known published¹⁹ and herein presented structures of
 417 LmUGP. Both the accessible surface area of the protein and the

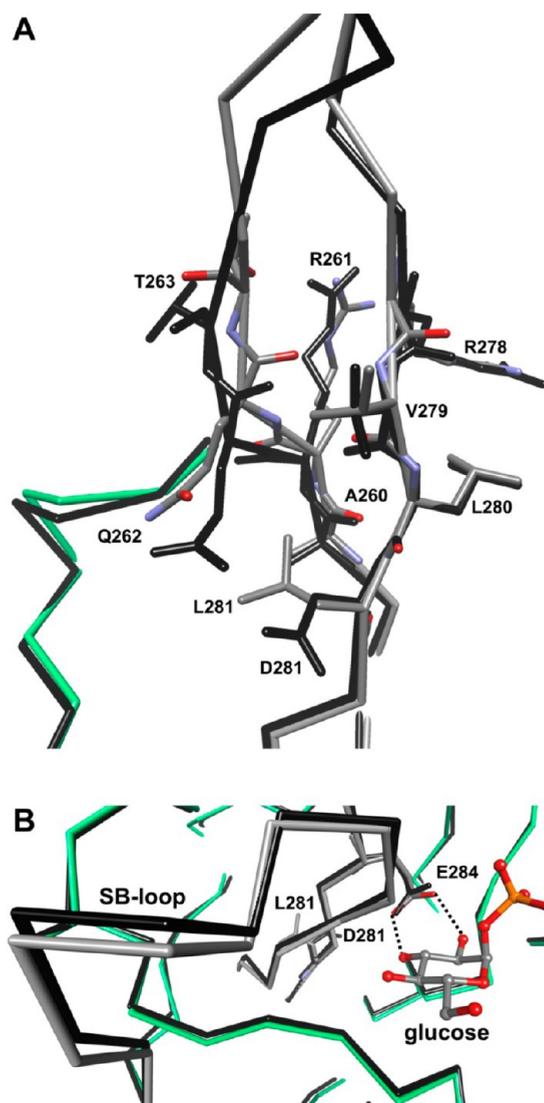


Figure 4. Superpositions of the wild-type (colored) and L281D mutant (black) LmUGP-UDP-Glc complexes. The mutation causes disturbances of the β -structure at the base of the handle (A), which results in the lack of closure in the SB loop region (B).

418 distances between key structural elements and the catalytic
 419 center were at their minimum in this state, whereas the
 420 torsional deformation of the β -sheet increased (Figure 2B).
 421 Compared with the UTP-state structure, the NB loop is shifted
 422 $\sim 2 \text{ \AA}$ closer toward the phosphate moiety, further bounding the
 423 nucleotide pocket (Figure 2C). The region R249–Y302 is in its
 424 closed state, providing maximal stabilization to the glucose ring.
 425 The amino group of the strictly conserved K380 is moved 5.6 Å
 426 toward the nucleotide, and an H-bond with the α -phosphate of
 427 UDP-Glc is formed. In the crystal structure, the sulfate ion
 428 occupies the position of the γ -phosphate in the LmUGP-
 429 dUpCcp complex. The identified magnesium ion coordinated
 430 sulfate, α -phosphate, and a water molecule. This water occupied
 431 the space between the α - and γ -phosphates and was additionally
 432 coordinated by an H-bond and electrostatic interactions with
 433 G86 and K95 of the NB loop. Superposition of the UTP and
 434 postreactive state structures suggested that in the prereactive
 435 state, the magnesium should be coordinated between the α -
 436 and β -phosphates of UTP (Supporting Information Figure S4).
 437 Of note, with the crystal structure of the postreactive state of 437

438 LmUGP, we present the first experimental structure in which
 439 the binding sites of all UGP products could be identified.
 440 Comparison of the crystal structures of LmUGP in the
 441 postreactive and the UDP-Glc-bound states revealed that
 442 dissociation of the pyrophosphate and magnesium leads to a
 443 significant change in the NB loop conformation (Figure 5). The

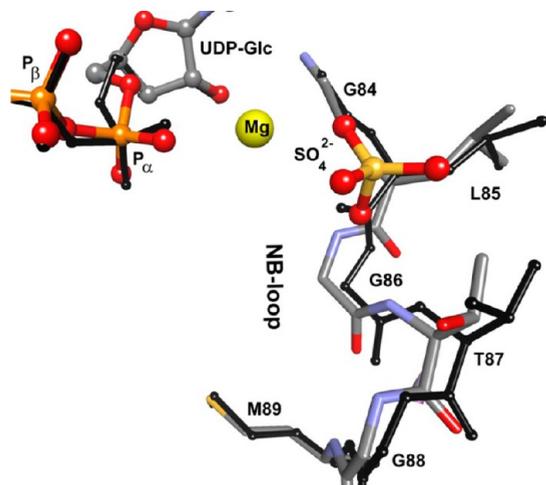


Figure 5. Experimental LmUGP crystal structures representing the postreactive (colored) and UDP-Glc (black) states. The superposition reveals conformational changes of the NB loop and phosphate moiety of UDP-Glc caused by the dissociation of pyrophosphate and Mg^{2+} .

444 G84–G88 residues and the adjacent G90–D93 region moved
 445 away from the active site, making it less compact. Because of
 446 the loss of the hydrogen bond with the γ -phosphate, the
 447 carbonyl group of the L85 peptide bond was flipped in the
 448 UDP-Glc state. As a result, the NB loop acquired an
 449 intermediate conformation between those of postreactive and
 450 apo states. In the crystal structure of the UDP-Glc state, the
 451 experimental electron density for this region was weaker and
 452 more disordered than that in the postreactive state (20%
 453 decrease in the signal-to-noise ratio for C_{α} atoms). A similar
 454 effect was observed for the SB loop residues, where
 455 destabilization led to a partial opening of the structure in the
 456 area of helix $\alpha 11$ (residues M293–N300) located at the end of
 457 the region R249–Y302. This destabilization effect is propagated
 458 from the NB to the SB loop via the shift of G88, which moves
 459 the conserved K422 (C-terminal domain) closer toward K255
 460 (SB loop) and, thus, increases the electrostatic repulsion
 461 between the two regions.

462 The flexible top part of the handle, including residues Y265–
 463 K277 that are located farther away from the protein globule and
 464 not stabilized by intramolecular interactions, is differently
 465 structured in the postreactive and the UDP-Glc states. The lack
 466 of electron density for Mg^{2+} in the UDP-Glc complex suggests
 467 that the ion dissociates together with the pyrophosphate. The
 468 phosphate moiety of UDP-Glc also undergoes a conformational
 469 change upon dissociation of Mg^{2+} and the PP_i (Figure 5). In
 470 the UDP-Glc state, the $C5^*-O5^*$ bond connecting the α -
 471 phosphate to the glucose ring is flipped 80° in comparison with
 472 the experimental geometry of the postreactive state. This
 473 change induces a shift of the K380 side chain, resulting in an
 474 increased distance between K380 and the oxygens of the α -
 475 phosphate and, thus, in the drop of affinity for UDP-Glc. All of
 476 the above effects reveal the mechanism of unlocking the

enzyme structure, which begins with the dissociation of the
 pyrophosphate.

Quantum Chemical Studies of LmUGP Catalysis. To study the electronic effects of UGP catalysis and identify the role of the active site residues in this process, we performed quantum chemical (QC) calculations of the LmUGP reaction pathway using the IRC procedure. The starting geometry approximation for the saddle point location search was generated using the coordinates of the protein part, the glucose ring, Mg^{2+} and water from the postreactive state complex, and the substrate coordinates from the structure of LmUGP·dUpCcp complex obtained in this work. After finding the transition state, the models of the pre- and postreactive states were obtained by descent forward and backward from the saddle point along the steepest descent path in mass-weighted Cartesian coordinates using the IRC method.⁴³ The overall protein conformation, the local structure of the active site residues, and the positions of nucleoside and glucose ring in the theoretical model of the LmUGP·UTP·Glc-1-P complex are very close to the experimental geometry of the postreactive state (r.m.s.d. = 1.2 Å; Figure 1C). This is in agreement with the principle of least motion in chemical reactions.⁴⁴ In the model, the phosphate moiety of UTP is structured similarly to that of the crystal structure of the LmUGP·dUpCcp complex (r.m.s.d. = 0.3 Å), whereas the phosphate group of Glc-1-P is oriented toward the α -phosphate of UTP, with a closest distance of 2.8 Å between the reagents. H191 and K380 stabilize the orientation of the Glc-1-P phosphate with four hydrogen bonds, ensuring an optimal position of the attacking oxygen with respect to the α -phosphate of UTP. In this position, the highest occupied molecular orbital (HOMO) localized on the attacking oxygen O_{3P} of Glc-1-P overlaps with the lowest unoccupied molecular orbital (LUMO) localized on the P_{α} atom of UTP, providing an electron exchange between the reactants (Figure 1C). Atomic partial charges resulting from Mulliken population analysis⁴⁵ reveal a significant electrostatic component to this interaction.

Importantly, the QC analysis showed that the magnesium ion coordinated by the oxygen atoms of the UTP α -phosphate affects both the partial charge distribution between the reacting atoms and the local geometry of the reaction center. Comparison of the QC prereactive state models with and without Mg^{2+} revealed that the Mulliken charges on P_{α}^{UTP} and $O_{3P}^{Glc-1-P}$ change from +1.4 and -0.7 in the presence of Mg^{2+} to +1.15 and -0.6 in the absence of Mg^{2+} , respectively. Thus, Mg^{2+} increases the polarization and electrostatic attraction of the reacting atoms. The involvement of $O_{1\alpha}$ and $O_{3\alpha}$ of the UTP α -phosphate in the coordination sphere of Mg^{2+} imposes a flattening effect of 10° on the α -phosphate geometry, rendering it closer to the transition state. In the presence of Mg^{2+} , the covalent bond $P_{\alpha}^{UTP}-O_{3\alpha}^{UTP}$ of the leaving group is elongated by 0.2 Å compared with that of the Mg^{2+} -free prereactive state, making dissociation along this bond easier. In contrast, however, the positioning, mutual orientation, and overall conformation of the reagents do not depend on Mg^{2+} , but are defined primarily by the protein matrix.

These data explain why in previous studies Mg^{2+} was shown to have a pronounced activation effect on UGPs⁹ but is dispensable for nucleotide binding.⁴⁶ Analysis of the potential energy profile along the UGP reaction coordinate revealed one barrier between the pre- and postreactive states. On the top of the barrier, the UTP α -phosphate acquires a trigonal bipyramidal configuration with a planar $P_{\alpha}O_3$ group and the

axial coordination sites occupied by the bridging $P_{\alpha}-O_{3\alpha}-P_{\beta}$ oxygen of UTP and the attacking oxygen from Glc-1-P phosphate (Figure 1D). The angle $O_{3\beta}^{Glc-1-P}-P_{\alpha}^{UTP}-O_{3\alpha}^{UTP}$ is increased from 160° in the prereactive state to 167° in the transition state. The movement of the attacking oxygen toward P_{α}^{UTP} is accomplished via rotational degrees of freedom of the Glc-1-P phosphate group. The elongation of the $P_{\alpha}^{UTP}-O_{3\alpha}^{UTP}$ bond leads to a shift of the $P_{\beta}-P_{\gamma}$ phosphates and Mg^{2+} toward the NB loop, inducing a small adjustment of the protein main chain around G86. K380, coordinating both Glc-1-P phosphate and UTP α -phosphate, shifts in the transition state toward the α -phosphate and stabilizes the planar conformation of the $P_{\alpha}O_3$ group. In addition, the length of the hydrogen bond between K95 and the $O_{3\beta}^{UTP}$ oxygen is decreased in the transition state, providing more stability to the position of the β -phosphate. The tightening of the contacts among K380, K95, and the phosphate moiety of UTP enhances the affinity of the enzyme for the transition state and helps to compensate for the increased atomic charges in the penta-coordinated intermediate. The positions of other residues, including H191 that coordinates the Glc-1-P phosphate, remain unchanged between the transition and prereactive states. Together, these results show that strictly conserved positively charged residues in the active site contribute to catalysis via electrostatic stabilization of the transition state, consistent with the loss of activity in K95A and K380A mutants.¹⁹

In agreement with an S_N2 associative mechanism, the formation of a penta-coordinated intermediate is followed by the stereochemical inversion of the α -phosphate, the breaking of the $P_{\alpha}^{UTP}-O_{3\alpha}^{UTP}$ bond, and generation of a leaving pyrophosphate group and UDP-Glc. The resulting theoretical model of the postreactive UGP·UDP-Glc· Mg^{2+} · PP_i complex and its crystal structure show close similarity (r.s.m.d. 1.2 Å) (Figure 1E). According to the model, the pyrophosphate binds into a pocket created by the NB loop residues G84–T87. There, it is coordinated by hydrogen bonds with the main-chain atoms of L85 and G86, the side chain of K95, and a number of van der Waals interactions. In the crystal structure, the positions of the γ - and β -phosphates are occupied by the sulfate ion and a water molecule, respectively. In both experimental and theoretical models, Mg^{2+} is coordinated between the α , β , and γ phosphates or their analogs in a similar way. Inversion of the α -phosphate and separation of PP_i induce small shifts of the H191, K380, and K95 side chains and the main chain of the NB loop. The frontier molecular orbitals in this state are localized on $O_{3\alpha}^{PP_i}$ (HOMO) and $P_{\alpha}^{UDP-Glc}$ (LUMO), which also represents the prereactive state of the backward reaction (Figure 1E). The distance between $O_{3\alpha}^{PP_i}$ and $P_{\alpha}^{UDP-Glc}$ is 0.5 Å longer compared with the reacting atoms of the UGP·UTP·Glc-1-P complex. The reaction pathway calculations predict the free energy barriers for the forward and backward reactions to be 20.8 and 22.2 kcal/mol, respectively (Supporting Information Figure S3). The corresponding values derived from the experimental specific activities V_{max} at a physiological temperature of 37 °C (Supporting Information Figure S2A,B) are 20.4 and 20.6 kcal/mol (experimental details are given in the Supporting Information). Thus, the reaction energy parameters produced in the calculated model are in a good agreement with the experimental kinetics data and reproduce the observed trend for the lower activation barrier in the forward reaction.

Our study demonstrates that the interaction of K95 with the β -phosphate is important to direct the pyrophosphate to its exit channel.

DISCUSSION

The data presented in this manuscript filled critical gaps in the knowledge of the LmUGP mechanism and allowed a complete reconstruction of the enzymatic cycle. In the forward reaction, the working cycle of LmUGP consists of five stages: closing (activated by UTP binding), locking (initiated by Glc-1-P binding), the enzyme-catalyzed chemical reaction, unlocking (activated by pyrophosphate dissociation), and opening (coupled with UDP-Glc dissociation).

LmUGP closing is activated by UTP, whose binding site already exists in the open state. The primary role of the nucleoside in UTP binding is supported by our structure of LmUGP H191L mutant, and the crucial role of the UTP γ -phosphate in the activation mechanism is elucidated by the structure of the LmUGP·dUpCcp complex. The activation effect of the γ -phosphate involves the large shift of the NB loop, which in its turn is bound to both the C-terminal domain and the seven-stranded β -sheet (Figure 2B). This shift causes conformational changes in these areas, particularly a torsional deformation of the β -sheet. The latter leads to a formation of the allosteric binding site for the second substrate. With this definition of the role of the γ -phosphate in the allosteric mechanism of LmUGP activation, our structure explains the previous findings² that UDP and UMP, lacking the γ -phosphate, are not able to activate LmUGP for the binding of the second substrate, Glc-1-P.

Binding of the second substrate initiates further changes in the LmUGP structure. The structural and kinetic data obtained with the mutants E284A and L281D strongly suggest that the UTP-induced torsional deformation of the β -sheet allows the glucose ring to be prepositioned in its binding site so that the interaction between the O2' and O3' hydroxyls of glucose and the carboxyl of E284 can occur. This interaction pulls the adjacent area of the handle, the SB loop and helix $\alpha 11$ toward the C-terminal domain, which stabilizes the Glc-1-P binding site, brings strictly conserved H191 and K380 to favorable positions for phosphate binding, and closes the LmUGP structure. Thus, E284 is playing a primary role in the process of glucose stabilization by LmUGP (as reflected by the >6-fold decrease in Glc-1-P affinity in the E284A mutant, Supporting Information Figure S2D) while the SB loop plays a secondary role. We defined this mechanism of the second substrate stabilization as “the lock mechanism” and the residues R249–Y302 as “the lock region” (Figure 1). The locking is facilitated by the base of the handle, which acts as a transducer of tensile force from E284 to the SB loop. The detailed allosteric mechanism of the lock was revealed by molecular dynamics simulations and confirmed by our kinetic and structural studies of LmUGP mutants.

Torsional deformation of the β -sheet and conformational changes in the lock area provide new insights into the mechanism of specific recognition of the second substrate by LmUGP.² Superposition of the LmUGP·UDP-Glc· Mg^{2+} · SO_4 complex (postreactive state) with the LmUSP·UDP-Gal complex⁴⁷ (a promiscuous UDP-sugar pyrophosphorylase) showed that the main steric hindrance preventing the binding of galactose by LmUGP is caused by the main chain of N306 ($\beta 11$). N306 belongs to the area where the torsional deformation of the β -sheet reaches its maximum. In LmUSP, 662

663 the torsional deformation of the β -sheet is of a lesser
664 magnitude, which leaves more space to accommodate
665 unspecific sugars. This observation indicates that the specificity
666 for the second substrate in nucleotidyltransferases can be
667 controlled via allosteric mechanisms.

668 Binding and stabilization of both substrates brings LmUGP
669 to the most compact state optimal for the catalytic reaction.
670 Our ab initio QM/MM calculations of the LmUGP reaction
671 pathway displayed the chemical conditions for the start of the
672 reaction elucidated the role of each residue in the catalytic
673 center during the reaction and explained the observed necessity
674 of Mg^{2+} for catalysis⁹ but not for the nucleotide binding.⁴⁶ The
675 LmUGP catalysis involves the proximity and orientation effect
676 provided by the active site protein matrix, bond strain and
677 electrostatic activation by the magnesium cofactor, and
678 electrostatic stabilization of the transition state by the positively
679 charged residues K380 and K95. The latter is also playing an
680 important role in directing the pyrophosphate to its exit
681 channel. With this, we provide a molecular explanation for the
682 previously hypothesized role of the positively charged active
683 site residues in guiding the PP_i release.¹¹

684 The structural models and reaction free energy barriers for
685 the forward and backward reactions obtained from the QC
686 calculations were in a good agreement with experimental data.
687 The position of Mg^{2+} in the experimental postreactive state
688 structure and in the QC models of LmUGP is similar to that in
689 the structure of bacterial guanosine-diphospho-D-mannose
690 pyrophosphorylase (GMP)⁴⁸ in complex with GTP (pdb code:
691 2X60) and to one of the two observed Mg^{2+} positions in the
692 complex of UGP from *C. glutamicum* with UDP-Glc⁴⁹ (pdb
693 code: 2PA4). It is interesting that in the latter structure as well
694 as in the complex of GMP with GDP-Man⁴⁸ (pdb code: 2X5Z),
695 the catalytically important Mg^{2+} occupies another position that
696 coincides with the position of the K380 amino group in
697 LmUGP (Supporting Information Figure S4). Despite the
698 different position, the Mg^{2+} in all structures includes the α -
699 phosphate group in its coordination sphere and imposes similar
700 distortions on the α -phosphate geometry as defined in
701 LmUGP. On the basis of this finding, it can be concluded
702 that, independent of the binding mode of Mg^{2+} in the different
703 nucleotidyltransferases, its mechanism of substrate activation
704 remains the same.

705 The high-resolution crystal structure of the postreactive state
706 showed for the first time the binding sites of all UGP products
707 and allowed revealing the mechanism of unlocking the enzyme
708 after the reaction. The unlocking is initiated by dissociation of
709 the pyrophosphate and Mg^{2+} ions. This causes the destabiliza-
710 tion of the NB loop and the lock region as well as a
711 conformational change of the UDP-Glc phosphate moiety, thus
712 activating the structure for closed-to-open transition. The full
713 opening of the enzyme should then be coupled with the
714 dissociation of UDP-Glc with the glucose part dissociating first.
715 It is of note to mention that we could confirm this reaction
716 sequence in a later structural study of human UGP (manuscript
717 in preparation).

718 The mechanisms of catalysis and allosteric regulation of
719 LmUGP as described in this work should significantly facilitate
720 investigations of the reaction cycles and allosteric regulation
721 networks of other nucleotidyltransferases that also follow
722 sequentially ordered bi-bi kinetics. Moreover, our data help
723 one to understand the coupling between global conformational
724 changes and events at the active site during the reaction of
725 these enzymes. This information would be particularly useful

for drug-design applications utilizing nucleotidyltransferases as
drug targets. Structural similarities of nucleotidyltransferases
make targeting their active sites likely to cause unwanted,
unspecific disruption of enzymatic pathways in the host. A
solution to this problem can be provided by employing
allosteric effects in the drug-design strategy, utilizing the
conformational control by nonconserved residues located far
from the catalytic center. A comprehensive description of the
enzymatic mechanism and allosteric effects would thus be an
important prerequisite for rational development of highly
specific allosteric regulators for these enzymes.

Finally, our structural and kinetic data on the L281D mutant
as well as computational analysis reveal the mechanism of
mechanical control of the chemical reaction in the active site of
LmUGP. This mechanism involves the tensile force trans-
duction between the strictly conserved residue E284 and the SB
loop via the β -structure of the handle. Decreasing the efficiency
of the force transduction by disturbing the β -structure leads to
the loss in specific activity of the enzyme, as demonstrated by
the L281D mutation. This illustrates how regulating the
efficiency of mechanical force transduction in allosteric chains
could be used to alter the catalytic activity in allosteric enzymes.
Small molecule compounds producing similar effects could be
used in drug design and biotechnological applications. The
concept of mechanical regulation of enzymatic and homoge-
neous chemical catalysts has attracted attention as a result of its
potential applications in biotechnology^{50–52} and new functional
materials.⁵³ Studying natural evolutionarily optimized mecha-
nisms of such regulation, as in the case of LmUGP, can have a
strong impact on the development of artificial mechanochem-
ical catalysts.

■ ASSOCIATED CONTENT

📄 Supporting Information

Crystallographic data and refinement statistics, experimental
electron density omit maps, kinetic data, reaction energy
diagram, and superposition of the active sites of nucleotidyl-
transferases. This information is available free of charge via the
Internet at <http://pubs.acs.org/>

Accession Codes

Coordinates for the structures LmUGPwt·UDP-Glc·Mg·SO₄,
LmUGP_{L281D}·UDP-Glc, LmUGP_{H191L}, and LmUGPwt·
dUpCp have been deposited in the Protein Data Bank with
the accession codes 4M2A, 4M2B, 4J18, and 4M28,
respectively.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-5115323705. Fax: +49-5115325966. E-mail:
Fedorov.Roman@mh-hannover.de.

Present Address

[†]Roche Diagnostics GmbH, Sandhofer Strasse 116, 68305
Mannheim, Germany.

Notes

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