Mycobacterial Ubiquitin-like Protein Ligase PafA Follows a Two-step Reaction Pathway with a Phosphorylated Pup Intermediate*  

Ethan Guth, Michael Thommen, and Eilika Weber-Ban  

From the Institute of Molecular Biology & Biophysics, ETH Zurich, CH-8093 Zurich, Switzerland

In *Mycobacterium tuberculosis*, the enzyme PafA is responsible for the activation and conjugation of the proteasome-targeting molecule Pup to protein substrates. As the proteasomal pathway has been shown to be vital to the persistence of *M. tuberculosis*, understanding the reaction mechanism of PafA is critical to the design of antituberculous agents. In this study, we have developed novel techniques to study the activity of PafA and have characterized fundamental features of the reaction mechanism. We show that PafA catalyzes a two-step reaction mechanism proceeding through a γ-glutamyl phosphate-mixed anhydride intermediate that is formed on the C-terminal glutamate of Pup before transfer of Pup to the substrate acceptor lysine. SDS-PAGE analysis of formation of the phosphorylated intermediate revealed that the rate of Pup activation matched the maximal steady-state rate of product formation in the overall reaction and suggested that Pup activation was rate-limiting when all substrates were present at saturating concentrations. Following activation, both ADP and the phosphorylated intermediate remained associated with the enzyme awaiting nucleophilic attack by a lysine residue of the target protein. The PafA reaction mechanism appeared to be noticeably biased toward the stable activation of Pup in the absence of additional substrate and required very low concentrations of ATP and Pup relative to other carboxylate-amine/ammonia ligase family members. The *bona fide* nucleophilic substrate PanB showed a 3 orders of magnitude stronger affinity than free lysine, promoting Pup conjugation to occur close to the rate limit of activation with physiologically relevant concentrations of substrate.

Tuberculosis remains a major world health epidemic as the leading cause of death among curable infectious diseases. *Mycobacterium tuberculosis*, the primary causative agent of the disease, evades elimination by the host immune response through intracellular persistence in alveolar macrophages. Intracellular persistence is promoted by strategies both to avoid activation of macrophage microbicidal activities (3–5) and to resist toxicological assault from reactive oxygen and nitrogen species deployed by macrophages (6). The *M. tuberculosis* proteasomal system plays a critical role in bacterial resistance to reactive nitrogen species both in *vitro* and in *vivo* (7) and makes use of a ubiquitin-like molecule, termed Pup (prokaryotic ubiquitin-like protein), to target proteins for degradation (8, 9). Although the pupylation system shares analogous features with the ubiquitin system, including post-translational processing, ATP-dependent activation of Pup, and isopeptide bond formation with lysine residues of target proteins, the chemistry of activation and conjugation is different, and the responsible enzymes do not share homology with ubiquitin-activating and ubiquitin-conjugating enzymes (8, 10, 11).

In *M. tuberculosis*, Pup is expressed with a C-terminal GGQ motif that first must be post-translationally processed by the enzyme Dop, which deamidates the C-terminal glutamine to glutamate (10, 11). The enzyme PafA utilizes ATP to activate deamidated Pup for conjugation to a lysine residue on the targeted protein (10) by way of the γ-carboxyl group of the Pup C-terminal glutamate (12). Conjugation of Pup to proteins can target them to the *M. tuberculosis* proteasome *in vitro* (13) and *in vivo* (14). Inhibition of the *M. tuberculosis* proteasomal system has gained attention due to its role in defending the bacteria against nitroxidative stress and its requirement for *M. tuberculosis* persistence in infected mice (15, 16). Direct inhibition of the *M. tuberculosis* proteasome has been shown to be bactericidal in non-replicating *M. tuberculosis* (17). PafA represents an attractive target to disable the *M. tuberculosis* Pup proteasomal system, as, unlike the proteasome itself, it does not share homology with its functionally analogous counterparts in the eukaryotic proteasomal system, and importantly, transposon mutants of PafA have already been shown to sensitize the bacteria to nitroxidative stress (7). A detailed understanding of the reaction mechanism of PafA will be critical for the design of effective inhibitors.

Bioinformatic investigations have shown that both PafA and Dop belong to the carboxylate-amine/ammonia ligase superfamily, a group that contains glutamine synthetase, γ-glutamylcysteine synthetase, and the amidotransferase Gat-CAB (18). The reaction mechanism for all of these family members is thought to follow a two-step reaction pathway in which the γ-glutamyl carboxylate is phosphorylated using ATP as the phosphate donor, followed by nucleophilic attack by either ammonia (glutamine synthetase, GatCAB) or the α-amino group of cysteine (γ-glutamylcysteine synthetase) (19–24). For the well studied members of the family, includ-

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† To whom correspondence should be addressed: Inst. of Molecular Biology & Biophysics, ETH Zurich, Schafmattstr. 20, CH-8093 Zurich, Switzerland. Tel.: 41-44-633-3678; Fax: 41-44-633-1229; E-mail: eilika@mol.biol.ethz.ch.
ing glutamine synthetase and γ-glutamylcysteine synthetase, kinetic characterization has revealed that the phosphorylated intermediate is efficiently formed only in the presence of the nucleophilic substrate (25, 26). Additionally, the phosphorylated intermediate itself has never been isolated.

In this investigation, we set out to characterize the fundamental mechanistic features of the PafA-catalyzed Pup conjugation reaction. We have previously shown that PafA turns over ATP to ADP at a 1:1 stoichiometry, with every deamidated Pup molecule being conjugated to a substrate, which suggests activation of Pup via phosphorylation (10). Utilizing a genetically encoded mutant of Pup with a C-terminal glutamate replacing glutamine (hereafter referred to as Pup-GGE), we demonstrate here that PafA follows a two-step reaction pathway with the formation of a phosphorylated Pup intermediate preceding the events of conjugation. Following activation, ADP and phosphorylated Pup-GGE remain associated with the enzyme. Formation of the activated intermediate does not depend on and is not made faster by the presence of the nucleophilic substrate and requires only low micromolar concentrations of Pup-GGE and ATP. The rate of conjugation is limited by binding of the nucleophilic substrate up to saturating levels, where the maximal steady-state rate of conjugation matches the rate of Pup activation as measured in isolation.

**EXPERIMENTAL PROCEDURES**

General Chemicals and Reagents—Unless noted otherwise, general chemicals were provided by Sigma. Radionucleotides were obtained from Hartmann Analytic (Braunschweig, Germany), with both [α-32P]ATP and [γ-32P]ATP provided at a specific activity of 111 TBq (3000 Ci)/mmol. Polyethyleneimine TLC plates were provided by VWR International. Chromatography columns were from GE Healthcare, and filtration and concentration supplies were from Millipore. All solutions were prepared in ELGA PURELAB purified water and ultrafiltered through a 0.45-μm filter before use.

Protein Expression and Purification—PafA, Pup-GGE, and PanB from M. tuberculosis were prepared as described (10) with the omission of EDTA from buffers in the PafA and Pup-GGE purification.

Monitoring ADP Production—All enzyme assays were carried out at room temperature (25 °C). Enzyme assays were performed in Buffer A (50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 20 mM MgCl₂, and 1 mM DTT). The standard reaction was carried out with an excess of substrates over PafA containing 20 μM Pup-GGE, 20 μM [α-32P]ATP (3.55 GBq (96 mCi))/mmol, and 60 μM PanB (monomer) or 80 mM lysine and was initiated by the addition of 1 μM PafA in a final volume of 20 μl. To monitor reaction progress, 2-μl aliquots of the reaction mixture were quenched by dilution into an equal volume of 20% SDS. Polyethyleneimine TLC plates were spotted with 0.5 μl of the quenched reaction mixture 2 cm from the edge of the plates and allowed to dry. Nucleotide species were separated using a mobile phase of 1 mM formic acid and 0.5 M LiCl. A Molecular Dynamics phosphorimaging screen (GE Healthcare) was exposed to the dried TLC plates for 1–2 h and subsequently developed using a STORM 820 phosphorimaging system (GE Healthcare). Images were analyzed using the Quantity One software package (Version 4.6.5, Bio-Rad) to determine the ratio of ADP produced to total nucleotide (ATP + ADP) for each reaction time point. This ratio was then multiplied by the known molar concentration of ATP added to the reaction mixture to determine the quantity of product formed per unit time. Mock reactions lacking PafA were performed in parallel, and the results were subtracted from PafA-containing reactions to account for spontaneous ATP hydrolysis during the reaction time course. In the absence of the nucleophilic substrates PanB and lysine, progress curves exhibiting burst phase kinetics were fit to a single-order exponential equation (Equation 1),

\[
y = a + b \cdot (1 - \exp^{-kt})
\]  
(Eq 1)

where \(a\) represents the \(y\) intercept, \(b\) represents the amplitude of the burst, and \(k_1\) represents the rate constant of the burst. The steady-state kinetic parameters were extracted from plots of the initial linear velocity of ADP production using at least six concentrations of the varied substrate. Data were fit to the standard Michaelis-Menten equation using the KaleidaGraph software package (Version 4.0.1, Synergy Software). All reactions were carried out at least three times and with at least two different PafA protein stocks.

Global Fitting of Data Sets—In the presence of the nucleophilic substrate, the data sets were globally fit using the Berkeley Madonna software package (Version 8.0.4) to a minimal reaction model, \(E + A \leftrightarrow EA \leftrightarrow EP + B \leftrightarrow EPB \leftrightarrow EQ \leftrightarrow E + Q\), where \(E\) represents PafA, \(A\) is a combined term reflecting the concentration of Pup-GGE and ATP (both present in 20-fold molar excess over PafA in the reaction), \(EP\) is the PafA-phosphorylated Pup-GGE complex, \(B\) is the nucleophilic substrate, and \(Q\) represents the associated products of the reaction.

Monitoring the Phosphorylation of Pup-GGE—Reactions consisting of 40 μM Pup-GGE and 20 μM ATP in Buffer A were initiated by the addition of 0.77 μM PafA. Reaction aliquots of 4 μl were quenched in an equal volume of SDS-PAGE loading buffer (300 mM Tris (pH 6.8), 10% SDS, 45% glycerol, 0.6 M DTT, and 0.015% bromphenol blue) and subsequently separated by SDS-PAGE. The gel was phosphorimaged as described above, and protein bands were developed by standard Coomassie Blue staining methods. Progress curves for the formation of phosphorylated Pup-GGE were fit to Equation 1.

Electrospray Ionization Mass Spectrometry of Phosphorylated Pup-GGE—To form the phosphorylated intermediate, Pup-GGE (4 μM) was mixed with PafA (3.4 μM), and ATP (20 μM) was added to start the reaction. After 20 min of incubation at 23 °C, protein samples (20 μl) were desalted with ZipTip C4 pipette tips (Millipore). Desalted samples were eluted from C4-coated pipette tips with 20 μl of 50% acetonitrile in water and loaded into the mass spectrometer. Spectra were recorded on a Micromass Q-TOF Ultima API system (Waters, Milford, MA). As a control, reactions were run in the absence of ATP, PafA, or both. Deconvolution of the resulting spectra was made difficult by the presence of ATP. Therefore,
analysis was confined to the $[M + 5H]^{5+}$ and $[M + 6H]^{6+}$ charged states.

Monitoring the Fate of ADP/Phosphorylated Pup-GGE—A 60-μl reaction containing 1.75 μM PafA, 10 μM Pup, and 20 μM ATP in Buffer A was incubated at 25 °C for 15 min. Following incubation, free ATP was removed by running the reaction mixture through a 700-μl protein desalting spin column (Pierce) equilibrated in Buffer A. The conjugation reaction was initiated by the addition of 5 μM PanB. Reaction aliquots of 5 μl were quenched in an equal volume of SDS-PAGE loading buffer, and the reaction was monitored by SDS-PAGE analysis following the mobility shift of the Pup-GGE-PanB conjugate. In a parallel series of experiments, $[\alpha-^{32}P]ATP$ or $[\gamma-^{32}P]ATP$ was incubated with the enzyme prior to spin column separation. Aliquots of 2 μl were subjected to scintillation counting before and after spin column chromatography to assess the retention of radiolabel in the reaction product. Controls in which PafA was omitted were used to correct for residual radionucleotide that was not efficiently removed by the procedure.

RESULTS

Burst Phase of ATP Hydrolysis Is Stoichiometric to the Concentration of PafA—To determine the events of ATP hydrolysis catalyzed by PafA, a radiological assay was developed that allowed the reaction to be monitored over the initial and subsequent turnovers of the enzyme. To observe the stoichiometry of product formation, 20 μM $[\alpha-^{32}P]ATP$ and Pup-GGE were titrated with PafA (0.5–1.5 μM). Aliquots were removed along the experimental time course and quenched with 20% SDS to stop the reaction, and the conversion of $[\alpha-^{32}P]ATP$ to $[\alpha-^{32}P]ADP$ was monitored by polyethyleneimine TLC and phosphorimaging. Consistent with previous investigations (10), in the absence of Pup-GGE, PafA did not display significant ATPase activity (Fig. 1, open squares), nor did Pup-GGE in the absence of PafA (Fig. 1, open circles). However, when both PafA and Pup-GGE were included in the reaction mixture, a burst of ADP production was observed that was stoichiometrically dependent on the concentration of PafA (Fig. 1, closed symbols) and could be readily fit to a single exponential with an observed rate constant of 0.99 ± 0.18 min$^{-1}$. Although the low concentrations of the PafA stock solution did not allow for an extensive titration series, when PafA was held in excess of Pup-GGE, the amplitude of the resulting burst was found to correspond to the concentration of Pup-GGE present (data not shown), confirming the dependence of the burst amplitude on the availability of both enzyme active sites and phosphorylation substrate.

Burst Phase of ATP Hydrolysis Correlates to the Production of Phosphorylated Pup-GGE—The observed correlation between the burst amplitude of ADP production and the concentration of PafA and Pup-GGE suggests that PafA can form an enzyme-bound phosphorylated intermediate in the absence of the nucleophilic substrate. On the basis of the stoichiometry of PafA to other carboxylate-amine/ammonia ligase family members, the stoichiometry of ATP consumption to Pup ligation (10), and the fact that Pup is ligated to substrate lysines via the γ-carboxyl of its C-terminal glutamate (12), we hypothesized that this intermediate was phosphorylated Pup-GGE. To investigate this possibility, we used $[\gamma-^{32}P]ATP$ and SDS-PAGE analysis to follow the covalent attachment of the terminal phosphate of ATP to Pup-GGE. Under these conditions, provided that the phosphorylated Pup-GGE species possesses sufficient stability to survive the reaction quench and subsequent SDS-PAGE, it would be expected that the radiolabel signal would overlap with the Pup-GGE protein band. When the gel was phosphorimaged and Coomassie Blue-stained in parallel, there was a clear overlap of the Pup-GGE protein band with a radiolabel signal that increased in intensity with incubation time while the Pup-GGE Coomassie Blue band intensity remained constant (Fig. 2A). As can be observed from the zero time point in which Pup-GGE was incubated with ATP for 5 min prior to the addition of enzyme to initiate the reaction, no incorporation of radiolabel into Pup-GGE was detected in the absence of PafA.

In addition to providing evidence for the PafA-dependent formation of a phosphorylated Pup intermediate, this result supports our assumption that quench of the reaction in SDS is sufficient to halt the reaction and liberate products that may be bound to the enzyme at the time of inactivation. The identity of this species as phosphorylated Pup-GGE was confirmed by electrospray ionization mass spectrometry, where an additional Pup species with an increase of 79 Da, corresponding to the addition of a phosphate group, was observed upon incubation of Pup-GGE, ATP, and PafA (Fig. 2C). No such Pup species was observed in equivalent time scale incubations of Pup-GGE alone or Pup-GGE in the presence of ATP. An analysis of the reaction time course demonstrated that the rate of increase of the radiolabeled signal in the Pup-GGE protein band, representing the production of the phosphorylated Pup-GGE species, could be fit to a single exponential with an observed rate constant of 1.2 ± 0.12 min$^{-1}$ (Fig. 2B), comparable with the rate constant governing the burst of ADP production.

ADP Remains Associated with PafA following Activation—To assess the fate of ADP following the activation reaction,
PafA was incubated with $\gamma$-$\text{P}^{32}$ATP and Pup-GGE and subsequently separated from unreacted nucleotide by size-exclusion spin column chromatography. Quantification of the $\gamma$-$\text{P}^{32}$-containing nucleotide coeluting with PafA by scintillation counting returned a value of 0.3 $\pm$ 0.1 mol of $\gamma$-$\text{P}^{32}$/mol of PafA. In contrast, a negligible amount of $\gamma$-$\text{P}^{32}$ was recovered with PafA in the absence of Pup-GGE, indicating that the binding of ATP to PafA alone is insufficiently tight and/or kinetically stable to survive spin column separation. As expected from the burst phase production of ADP, analysis of the recovered sample by polyethyleneimine TLC demonstrated that the recovered nucleotide was $\gamma$-$\text{P}^{32}$ADP (data not shown). Thus, ADP remained bound to PafA following the completion of the activation reaction. Parallel experiments utilizing $\gamma$-$\text{P}^{32}$ATP demonstrated a stoichiometrically equivalent recovery of $\gamma$-$\text{P}^{32}$/mol of PafA, confirming the stoichiometric relationship between production of ADP and that of phosphorylated Pup-GGE. This result supports a reaction model wherein phosphorylated Pup-GGE and ADP are retained on the enzyme in a stable fashion following activation.

**Steady-state Characterization of the PafA Activation Reaction**—As a member of the amine/ammonia superfamily, PafA shares many family-conserved active-site residues responsible for ATP binding and $\gamma$-glutamyl kinase activity (18). To evaluate the steady-state kinetic parameters governing the $\gamma$-glutamyl kinase activity of PafA, ATP or Pup-GGE was held at a fixed concentration of 20 $\mu$M while varying the concentration of the respective substrate from 0.5 to 20 $\mu$M. The initial velocity of ADP formation was plotted against the concentration of the varied substrate, and the Michaelis parameters were determined (Table 1). A surprising result of these experiments was the relatively low determined $K_m$ values, in comparison with other members of the carboxylate-amine ligase family, of 8.7 $\pm$ 3.7 $\mu$M for ATP and 1.4 $\pm$ 0.24 $\mu$M for Pup-GGE.

**Phosphorylated Pup-GGE Is Competent for Conjugation to the Nucleophilic Substrate**—For the production of phosphorylated Pup-GGE (as measured in isolation) to have relevance for the overall reaction, the activated intermediate must be kinetically competent. To investigate the competence of the phosphorylated Pup-GGE intermediate as a substrate for the

*TABLE 1*  
**Apparent Michaelis constants determined from steady-state assays at 25 °C and pH 7.4**

| Variable substrate | Fixed substrate(s) | $K_m$ $\mu$M |
|--------------------|--------------------|-------------|
| ATP (0.5–20 $\mu$M) | Pup-GGE (20 $\mu$M) | 0.0087 ± 0.0037 |
| Pup-GGE (0.5–20 $\mu$M) | ATP (20 $\mu$M) | 0.0014 ± 0.0002 |
| PanB (0.5–60 $\mu$M) | Pup-GGE (20 $\mu$M) and ATP (20 $\mu$M) | 0.0142 ± 0.0076 |
| Lysine (1–80 mM) | Pup-GGE (20 $\mu$M) and ATP (20 $\mu$M) | 22.0 ± 10.2 |

*FIGURE 2. Time course of Pup-GGE phosphorylation.* PafA (0.77 $\mu$M) was mixed with 40 $\mu$M Pup-GGE and 20 $\mu$M $\gamma$-$\text{P}^{32}$ATP to initiate the reaction. At the indicated time points, 4-µl reaction aliquots were mixed with 4 µl of 6× SDS-PAGE loading buffer to quench the reaction. The quenched reactions were subjected to 15% SDS-PAGE and phosphorimaged and stained in parallel. A, comparison of the Coomassie Blue-stained Pup-GGE band (left panel) and the phosphorimage of the same gel (right panel). The asterisk denotes the position of residual contaminants in the PafA preparation. B, time course of Pup-GGE phosphorylation as determined by densitometry of the phosphorylated Pup-GGE band. C, mass spectrometry of Pup-GGE and ATP in the presence and absence of PafA. The [M + 5H]$^+$ and [M + 6H]$^+$ data sets are rendered in black and gray, respectively. To convert between the mass of the charged fragment and the mass of the full-length protein, subtract 1 from the axis value and multiply the difference by the charge state of the fragment. The molecular mass of the Pup-GGE construct used in these studies, as confirmed by mass spectrometry in the absence of ATP, is 7089.4 Da. *Rel.*, relative.
Mechanism of M. tuberculosis PafA

**Figure 3.** Conjugation of activated Pup-GGE from the preformed enzyme-intermediate complex to the substrate PanB. PafA (1.75 μM) was incubated with Pup-GGE (5 μM) and ATP (20 μM) for 15 min at 25 °C. In parallel control reactions, ATP was excluded from the reaction mixture. The reaction mixture was cleared of ATP by desalting spin columns, and the conjugation reaction was initiated by the addition of 5 μM PanB. A, SDS-polyacrylamide gel showing the mobility shift of PanB when conjugated to Pup-GGE over the reaction time scale. B, rate of Pup-PanB formation as determined by densitometric analysis of the Pup-PanB band in relation to the total concentration of PanB. Pup-GGE was poorly stained by Coomassie Blue and was not expected to contribute to the density of the Pup-PanB band at the concentrations under which the assay was performed.

Conjugation reaction, a preformed enzyme-intermediate complex was isolated by size-exclusion spin column chromatography and mixed with the cognate nucleophilic substrate PanB (5 μM). Under these conditions, the transfer of Pup-GGE to PanB was limited by the concentration of the recovered activated complex (estimated to be ~1 μM) because ATP had been removed from the reaction. As PanB was in excess of the recovered complex, pseudo first-order conditions applied, and a single exponential was expected that relates product formation to time. As shown in Fig. 3A, the isolated complex of PafA and phosphorylated Pup-GGE supported the transfer of Pup-GGE to the substrate nucleophile. The rate of production of PanB-Pup was readily fit to a single exponential with an observed rate constant of 0.19 ± 0.04 min⁻¹ (Fig. 3B).

**Rate of Activation Matches the Steady-state Rate of Turnover**—As indicated by the robust fit of single-exponential progress curves to the data in Fig. 1, ADP production was effectively halted after activation of Pup-GGE in the absence of the substrate nucleophile. As the isolated enzyme intermediate supports conjugation, it would be expected that the addition of the substrate nucleophile to a preformed enzyme-intermediate complex in the presence of excess Pup-GGE and ATP would clear the active site of phosphorylated Pup-GGE and ADP and allow subsequent rounds of catalysis to take place. Furthermore, if the events of conjugation were sufficiently fast, it would be expected that the addition of nucleophilic substrate would allow the reaction to take place at a maximal rate governed by the rate of activation. To observe both the events of activation and conjugation in isolation, we delayed the introduction of the nucleophilic substrate and observed the first round of ADP production, which could be fit to a single exponential with an observed rate constant of 1.1 ± 0.1 min⁻¹ (Fig. 4). When saturating quantities of the model substrate lysine were added, a linear steady-state production of ADP was observed at a rate of 0.95 ± 0.1 min⁻¹. The same linear production of ADP was observed when saturating quantities of either the bona fide nucleophile PanB or free lysine were incubated with ATP and Pup-GGE, and the reaction was initiated by the addition of PafA (Fig. 5). Thus, the overall steady-state rate of conjugation is matched by the rate of Pup-GGE activation as measured in isolation.

**Overall Reaction Shows Explicit Dependence on the Concentration of the Nucleophilic Substrate**—To further define the conjugation reaction, titration series using either the bona fide nucleophilic substrate PanB at concentrations of 2–60 μM or the model substrate lysine at concentrations of 1–80 mM were performed. In these reactions, ATP and Pup-GGE were present at saturating concentrations and in 20-fold excess of the concentration of PafA. All substrates were present prior to the addition of PafA to initiate the reaction. Titration series using either PanB or lysine revealed identical kinetic profile shapes (Fig. 5, A and B). Below saturation, a clearly defined burst of ADP production was followed by a linear phase, indicating that, under these conditions, the rate of activation proceeds faster compared with conjugation. The linear phase of ADP production followed hyperbolic dependence on substrate and, at the highest concentrations of substrate measured, assumed a linear extrapolation to the origin of the reaction. The maximal rate of ADP production measured with saturating concentrations of bona fide or model nucleophilic substrate matched the burst phase rate of ADP production as measured in the absence of the substrate nucleophile, indicating that the maximal steady-state rate of the reaction is limited by the activation of Pup. The rate dependence of the linear phase of ADP production on substrate concentration could be fit to yield $K_m$ values of 14.2 ± 7.6 μM and 22 ± 10.2 mM for PanB and lysine, respectively (Table 1).
Progress curves from the titration series were globally fit to a simplified model as described under “Experimental Procedures.” As an initial approximation, the initial ground state assumption, global fitting of both data sets returned values of $k_{cat}/K_m$ for PanB and lysine of $7 \times 10^{-2}$ and $4.5 \times 10^{-5} \text{M}^{-1} \text{min}^{-1}$, respectively. Although these parameters are not well defined by the model, the potential that the rate of conjugation is limited by binding of the nucleophilic substrate has been isolated from glutathione synthetase and succinyl-CoA synthetase systems (28–30).

That PafA produces phosphorylated Pup-GGE in the absence of the nucleophilic substrate does not necessarily place this species on-pathway in the overall reaction. Other members of the amine/ammonia ligase family have demonstrated partial reactions in the absence of the nucleophilic substrate that are kinetically off-pathway. For example, $\gamma$-glutamylcysteine synthetase has been shown to generate pyrrolidine-2,5-dicarboxylate (the presumed cyclization product of $\gamma$-glutamyl phosphate) but at a rate of $0.5\%$ of the conjugation reaction ($0.19 \pm 0.04 \text{min}^{-1}$) was significantly less than the rate of the reaction at saturating concentrations of the nucleophilic substrate. Additional parameters governing the rate of conjugation itself as well as product release were likewise not well defined by the model but were required to be at least an order of magnitude greater than the rate of activation to fit the observed data, further supporting a model of the overall reaction in which activation is rate-limiting for catalysis.

**DISCUSSION**

Mechanistic investigations of other members of the carboxylate-amino/ammonia ligase family such as glutamine synthetase, $\gamma$-glutamylcysteine synthetase, and GatCAB have indicated that their reactions proceed by a two-step mechanism with formation of a $\gamma$-glutamyl phosphate species preceding attack by a nucleophilic amine. However, for those enzymes, isolation of the phosphorylated intermediate has not been observed. In glutamine synthetase and $\gamma$-glutamylcysteine synthetase systems, rapid cyclization of the intermediate due to nucleophilic attack by the glutamate’s own amine group results in the production of pyrrolidone carboxylate. In the GatCAB system, where the C-terminal carboxyl group of glutamate is covalently bound to tRNA$_{Gln}$, the analogous species, Pyr-tRNA$_{Gln}$, is formed (23, 27). Here, we present compelling evidence for the formation of the activated phosphorylated Pup-GGE intermediate based on incorporation of radiolabeled phosphate into the Pup-GGE protein (Fig. 2A) and on mass spectrometry (Fig. 2C). This conclusion is further supported by the equivalence of measured rates of phosphate incorporation into Pup-GGE and ADP production in parallel experiments (Figs. 1 and 2B). The stability of the phosphorylated Pup-GGE species in the PaFA reaction is likely due to the chemical arrangement of the activated glutamate in which the amino functional group is involved in a peptide bond with the penultimate glycine of the C terminus, precluding internal cyclization. In support of this argument, chemically analogous acyl phosphate intermediates have been isolated from glutathione synthetase and succinyl-CoA synthetase systems (28–30).

That PafA produces phosphorylated Pup-GGE in the absence of the nucleophilic substrate does not necessarily place this species on-pathway in the overall reaction. Other members of the amine/ammonia ligase family have demonstrated partial reactions in the absence of the nucleophilic substrate that are kinetically off-pathway. For example, $\gamma$-glutamylcysteine synthetase has been shown to generate pyrrolidone carboxylate (the presumed cyclization product of $\gamma$-glutamyl phosphate) but at a rate of $0.5\%$ of the conjugation reaction (22, 26, 31). Additionally, in glutamine synthetase, the formation of a $\gamma$-glutamyl phosphate intermediate has been shown to be accelerated by the presence of the nucleophilic substrate such that the rate of the activation reaction as measured in isolation is almost an order of magnitude slower than the true rate of activation in the full biosynthetic reaction (25). In contrast, the production of the phosphorylated intermediate by PafA occurs at a rate that is identical to the maximal rate of the overall reaction as measured under saturating conditions of the nucleophilic substrate (Fig. 4). Size-exclusion isolation
Mechanism of M. tuberculosis PafA

experiments revealed that the stoichiometric equivalences of ADP and phosphorylated Pup-GGE coelute with PafA following ATP hydrolysis, arguing that the activated intermediate and ADP remain bound to the enzyme awaiting the arrival of the nucleophilic substrate. Further supporting this conclusion is the observation that the stoichiometry and kinetics of the burst of ADP production are unaltered in the presence of sub-saturating concentrations of the bona fide nucleophilic substrate PanB or the model substrate lysine (Fig. 5, A and B). Additionally, the isolated enzyme-intermediate complex promotes conjugation of Pup-GGE to PanB at the same rate as the full biosynthetic reaction given the same concentration of nucleophilic substrate (Figs. 3B and 5A). However, as the rate of activation becomes collinear with the rate of conjugation under saturating conditions of nucleophilic substrate, and thus obscured, it cannot be ruled out that this reaction proceeds by way of the quaternary complex of PafA, ATP, Pup-GGE, and substrate when all substrates are saturating, as has been observed for glutamine synthetase (25, 32, 33). In either case, the equivalence of the rate of activation as measured in isolation to the rates of conjugation at saturating conditions of genuine or model substrate suggests that activation is rate-limiting to the overall reaction under saturating conditions of substrate.

The equivalence of the kinetic profiles with both PanB and free lysine (Fig. 5 A and B) argues that both substrates proceed along the same reaction pathway, which is supported by the finding that both free lysine and the lysine side chain of PanB are conjugated to Pup by the Pup γ-carboxyl group to the ε-amino group of lysine (12). The only apparent difference between the activities of the two substrates is reflected in the $K_m$ terms, resulting in a specificity constant for the bona fide substrate 3 orders of magnitude higher than that of the model substrate. Although the experimental conditions were not sufficient to constrain individual values of the microscopic rate constants along the reaction pathway, this conclusion is reinforced by qualitative assessment of the results from global fitting, where the primary parametric difference between PanB and lysine data sets was confined to binding of the nucleophilic substrate. Our data support a model of the PafA reaction mechanism where the initial event of activation of Pup-GGE is unaffected by the presence of the substrate nucleophile and where, under saturating conditions, the rate of the reaction is ultimately limited by the rate of activation. Our data also suggest that specificity elements of the bona fide nucleophilic substrate serve to promote binding to the enzyme and/or enzyme-intermediate complex to allow the subsequent steps of conjugation to take place. Further investigations will be necessary to define the specificity determinants within the substrate that promote its utilization by PafA in the conjugation reaction and to answer the questions of when in the reaction sequence the substrate nucleophile binds to PafA and in which order products are released.

Despite a paucity of general sequence conservation, key residues included in the ATP- and glutamate-binding pocket are present in all members of the carboxylate-amine/ammonia ligase family, including the newly defined member PafA (18). Importantly, PafA variants in which these key residues have been individually replaced fail to support pupylation in vivo and in vitro (11, 34). This conservation of residues is reflected in the general conservation of mechanistic features found among the well studied members of this family, including the formation of a γ-glutamyl phosphorylated intermediate in a two-step reaction pathway leading to product formation. However, PafA demonstrates a number of novel mechanistic features that distinguish it from other members of the carboxylate-amine/ammonia ligase family. As described above, the $K_m$ values for ATP and the glutamate-containing substrate are orders of magnitude lower than those found in other members of the family. In PafA, the stoichiometric and stable formation of the phosphorylated intermediate appears to be independent of the presence of the nucleophilic substrate. This intermediate is kinetically competent to participate in conjugation at a rate defined by the concentration of the nucleophilic substrate. Interestingly, in glutamine synthetase, mutations of conserved active-site residues have been observed to differentially affect the phosphoryl transfer and biosynthetic reactions (35–37). In PafA, mechanistic segregation of activation and conjugation activities may be a product of redefined roles for conserved active-site residues.

The low $K_m$ for nucleotide in particular suggests that PafA may be insensitive to the energy charge of the cell, relying on alternative mechanisms for regulation of activity (38). The observed 3 orders of magnitude difference in the specificity constant between bona fide and model substrates of conjugation suggests that regulation of activity may be provided by the concentration of the nucleophilic substrate possessing the correct specificity determinants. Such a mechanism would provide a link between the activity of PafA and the availability of the target proteins requiring degradation by the proteasome.

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Mechanism of M. tuberculosis PafA

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