Elucidating the Role of Conserved Glutamates in H⁺-pyrophosphatase of *Rhodospirillum rubrum*

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H⁺-pyrophosphatase (H⁺-PPase) catalyzes pyrophosphate-driven proton transport against the electrochemical potential gradient in various biological membranes. All 50 of the known H⁺-PPase amino acid sequences contain four invariant glutamate residues. In this study, we use site-directed mutagenesis in conjunction with functional studies to determine the roles of the glutamate residues Glu₁⁹⁷, Glu₂₀², Glu₅₅⁰, and Glu₆₄⁹ in the H⁺-PPase of *Rhodospirillum rubrum* (R-PPase). All residues were replaced with Asp and Ala. The resulting eight variant R-PPases were expressed in *Escherichia coli* and isolated as inner membrane vesicles. All substitutions, except E₂₀²A, generated enzymes capable of PPi hydrolysis and PPi-energized proton translocation, indicating that the negative charge of Glu₂₀² is essential for R-PPase function. The hydrolytic activities of all other PPases, except E₂₀²A, were impaired at low Mg²⁺ concentrations but were only slightly affected at high Mg²⁺ concentrations, signifying that catalysis proceeds through a three-metal pathway in contrast to wild-type R-PPase, which employs both two- and three-metal pathways. Substitution of Glu₁⁹⁷, Glu₂₀², and Glu₆₄⁹ resulted in decreased binding affinity for the substrate analogues aminomethylendiphosphonate and methylendiphosphonate, indicating that these residues are involved in substrate binding as ligands for bridging metal ions. Following the substitutions of Glu₅₅⁰ and Glu₆₄⁹, R-PPase was more susceptible to inactivation by the sulfhydryl reagent mersalyl, highlighting a role of these residues in maintaining enzyme tertiary structure. None of the substitutions affected the coupling of PPi hydrolysis to proton transport.

Proton pumping pyrophosphatase (H⁺-PPase) is an integral membrane protein that utilizes the energy released upon the hydrolysis of PPi to transport protons across the membrane against the electrochemical potential gradient (1–3). H⁺-PPases represent a distinct class of ion translocases with no sequence similarity to ubiquitous ATP-energized pumps such as Escherichia coli V- or P-type ATPases or ABC transporters (4). The distribution of H⁺-PPase among the species is wide, but sporadic. H⁺-PPase has been identified in most plants, some algae, protists, bacteria, and Archaea, but not in mammals. In prokaryotic species, H⁺-PPase resides in the cytoplasmic membrane and pumps protons away from the cytoplasm, whereas in eukaryotic species the enzyme acidifies internal organelles such as vacuoles in plants (2, 3) and acidocalcisomes in protozoa (5). Moreover, H⁺-PPases have recently been identified in the plasma membranes of protozoa (6, 7). Both hydrolytic and proton translocation activities are associated with a single polypeptide of 66–90 kDa (8–11), which possibly forms a dimer (10, 12). H⁺-PPase is a highly hydrophobic protein, as evident from the 14–16 transmembrane spans predicted by computer modeling (Fig. 1). Sequences from different species display at least 30% identity, and most of the 60 conserved residues are clustered in three hydrophobic regions comprising cytosolic loops that probably form the active site. These residues include four Glu residues (Fig. 1) that are present in all 50 of the known H⁺-PPase sequences.

H⁺-PPase acts specifically on PPi and requires Mg²⁺ for activity (13–15). Mg²⁺ binds and activates both free enzyme and PPi. Some H⁺-PPases additionally require potassium for activity (2, 16). A common property of H⁺-PPases that distinguishes them from their soluble counterparts is high sensitivity to competitive inhibition by aminomethylendiphosphonate (AMDP) and relative insensitivity to inhibition by fluoride (17). Moreover, H⁺-PPases are highly sensitive to sulfhydryl reagents, such as mersalyl and N-ethylmaleimide, and the carboxyl group reagent dicyclohexylcarbodiimide (2).

The development of expression systems for plant H⁺-PPases in yeast Saccharomyces cerevisiae (9) and bacterial H⁺-PPases in *Escherichia coli* (18) has opened the way for site-directed mutagenesis of these proton pumps. This approach was successfully employed to identify membrane-embedded charged residues contributing to dicyclohexylcarbodiimide binding in H⁺-PPases (19), reactive Cys residues (18, 20), and those residues that determine the potassium requirement of H⁺-PPases (16). The role of conserved residues possibly located in the cytosol is more difficult to analyze, because their substitution...
results in an inactive enzyme (21). Here we perform site-directed mutagenesis of all four conserved glutamate residues, Glu197, Glu202, Glu550, and Glu649, in the H⁺-PPase of Rhodospirillum rubrum. All of the residues were functionally substituted with aspartates or alanines except Glu202, which was only replaceable by aspartate. All of the glutamates appeared to be essential for catalytic efficiency at low Mg²⁺ concentrations. Our data indicate that Glu197 and Glu202 contribute to substrate binding, whereas Glu550 and Glu649 control R-PPase conformation.

EXPERIMENTAL PROCEDURES

Plasmid Construction and R-PPase Expression—The construct comprising the full-length R-PPase gene (22) cloned into the pET22b(−) vector under the control of the T7 lac promotor was described previously (18). Mutagenesis was performed by an overlapping PCR technique using the Stratagene QuickChange™ kit and the primers listed in Table I. All mutations were verified by DNA sequencing. Wild-type and variant R-PPases were expressed in E. coli C43(DE3) cells, and inner membrane vesicles (IMVs) were isolated as described previously (18). Protein concentrations in E. coli IMV were estimated using the Bradford assay (23).

Activity Measurements—PP, hydrolysis was assayed by continuously recording P₂ liberation with an automatic P₂ analyzer (24) at 25 °C. Reactions were performed in an initial volume of 25 μl including pH buffer, calculated amounts of MgCl₂ and Na₄PPi, and 5–50 μl of IMV suspension. The reaction was initiated by the addition of IMVs in simultaneous addition of MgCl₂ and Na₄PPi in all other measurements. P₂ liberation was monitored for 3 min. The following 0.1 μl buffers were used at a pH equal to their pH₅₀ values: MES, pH 6.1; MOPS, pH 6.9; MOPS, pH 7.2; TES, pH 7.4; TAPS, pH 7.5; HEPES, pH 7.8; Tricine, pH 8.1; TAPS, pH 8.4; CAPSO, pH 9.6, and CAPS, pH 10.4. The pH was adjusted using KOH, and the indicated amounts of ECTA were added as follows: 50–100 μM, pH 6.1–8.1; 10 μM, pH 8.4; and 1 μM, 9.6–10.4. The resulting ionic strength of the buffers was 0.1 M, and the K⁺ concentration was adjusted using KOH, and the indicated amounts of EGTA were added.

The pH dependence of k₇₅ was fitted to Equation 4,

\[ k₇₅ = \frac{k_{₇₅}}{1 + [H⁺]/K₇₅} \]

where k₇₅ is the pH-independent value of k₇₅, and K₇₅ is the ionization constants of essential base and acid, respectively.

The time courses of the slow phase of R-PPase inactivation by mersalyl were fitted to Equation 5,

\[ A = A₀e^{-k(t)/M₉₅} \]

where A₀ is the residual activity observed after the “instant” inactivation phase, A is activity at time t, k is the second-order rate constant, and [Mers] depicts mersalyl concentration. The dependence of k on Mg²⁺ concentration is estimated by Equation 6,

\[ k = \frac{k_{₇₅}M₉₅}{M₉₅ + k_{₇₅M₉₅}} \]

where k₇₅ and k₇₅M₉₅ are the second-order rate constants for inactivation at 0 and infinite Mg²⁺ concentrations, respectively, and KM₉₅ refers to the dissociation constant for the R-PPase-Mg²⁺ complex (26).

Competitive inhibition of PP, hydrolysis by diphosphonate was analyzed with Equation 7,

\[ v/E₀ = \frac{k_{₇₅}}{1 + K_{₇₅}(1 + [U]/[M₉₅])} \]
where \([I]\) is the diphosphonate concentration and \(K_i\) is the inhibition constant. The \(\text{Mg}_2\text{PP}\) concentration was maintained at 50 \(\mu\text{M}\) in this experiment. The parameters, \(k_{\text{cat}}^\text{app}\) and \(K_i\), were constrained to the values derived from the dependence of \(v/[E_i]\) on \([\text{Mg}_2\text{PP}]\) in the absence of diphosphonates. The equation was fitted to data using the programs SCIENTIST 2.01 (MicroMath) and Origin 6.1 (OriginLab).

**RESULTS**

Hydrolytic and Proton-pumping Activities of R-PPase Variants—Each of the four conserved glutamate residues in R-PPase was individually substituted with aspartate and alanine, and the resulting variants were expressed in *E. coli* C43(DE3). Western analysis of *E. coli* IMVs with R-PPase antibodies (22) revealed that the expression levels of all the variants were similar to that of the wild-type enzyme (data not shown). The hydrolytic activities of R-PPase variants were assayed in IMVs at saturating substrate and \(\text{Mg}^{2+}\) concentrations. Specifically, the E197D, E197A, E202D, E550D, E550A, E649D, and E649A variants displayed 60, 60, 35, 50, 50, 45, and 20% of the wild-type R-PPase activity, respectively. However, following E202A substitution, activity declined to a level over 5% of wild-type R-PPase activity. Interestingly, the replacement of Glu197 or Glu550 by alanine did not induce a further decrease in activity as compared with substitution with aspartate. More-
tory fit. Therefore, we assumed that $k_a = k_h$, as was shown previously for authentic R-PPase isolated from *R. rubrum* chromatophores (26). In contrast, setting $k_a = 0$ was required to obtain an acceptable fit for all variant R-PPases.

For the three active Glu→Ala variants (E197A, E550A, and E649A), the $k_a^o/K_{M,mers}$ values were lower by a factor of 3.5–9 at 0.1 mM Mg$^{2+}$ as compared with that at 2 mM Mg$^{2+}$ (data not shown). In this respect, alanine variants were similar to aspartate mutants (Fig. 3B). The absolute values of $k_a^o/K_{M,mers}$ were similar to those measured for the corresponding Glu→Asp variants.

**Dependence of $k_a^o$ on pH**—The pH dependences of $k_a^o$ for the wild-type and variant R-PPases were bell-shaped (data not shown), indicating that one basic and one acidic group is necessary for the substrate conversion step. In this respect, R-PPase is similar to the H$^+$-PPase of the plant *Vigna radiata* (25). The two corresponding $pK_a$ values, along with the pH-independent values of the catalytic constant, $k_{h,init}$, derived from these dependences with Equation 4 are listed in Table II.

The effects of the mutations on the $pK_a$ values were insignificant except for the observed increase in $pK_a$ by 0.6 in the E202D variant, the increase in $pK_a$ by 0.6, and the decrease in $pK_a$ by 1.1 in the E649D variant. Interestingly, the pH-independent $k_a$ values for the E649D variant and the wild-type enzyme were similar within the combined experimental error, indicating that the low $k_a^o$ value for the E649D variant observed at pH 7.2 is due to the effect of the substitution on the $pK_a$ of ionizing groups rather than to intrinsic catalytic efficiency.

**Mersalyl Inactivation and Mg$^{2+}$ Binding**—R-PPase contains three mersalyl-reactive Cys residues (18). Modification of Cys$^{222}$ occurs nearly instantly and decreases enzyme activity slightly (typically by $\sim 10\%$), whereas modification of Cys$^{195}$ and Cys$^{573}$ can be resolved in time and render R-PPase completely inactive. Cys$^{195}$ and Cys$^{573}$ modifications are protected by Mg$^{2+}$ binding. To compare the effects of the mutations on mersalyl inactivation, an additional C185G substitution corresponding to the naturally occurring isomerase was introduced to block Mg$^{2+}$-dependent inactivation resulting from Cys$^{195}$ modification (18). The C185G substitution did not affect the hydrolytic and transport activities of the variant and wild-type R-PPase (data not shown) but made the slower inactivation step a first-order reaction at a fixed mersalyl concentration (Fig. 4), thus facilitating quantitative analysis.

The values of the second-order rate constants for inactivation in the absence of Mg$^{2+}$ ($k_{E,mers}$) and at saturating Mg$^{2+}$ concentrations ($k_{EM,mers}$) derived from the time-course experiments depicted in Fig. 4 were similar for wild-type, E197D, and E202D variants (Table II). In contrast, the E550D and E649D substitutions increased the $k_{E,mers}$ values by factors of 20 and 4, respectively. In all cases, Mg$^{2+}$ afforded substantial protection against mersalyl inactivation ($k_{E,mers} > k_{EM,mers}$). In the E550D variant, the $k_{E,mers}/k_{EM,mers}$ ratio decreased by a factor of 2, which indicates that, upon Mg$^{2+}$ binding, the conformational change that inhibits the reactivity of Cys$^{573}$ in R-PPase toward SH reagents becomes partially compromised.

The values of the dissociation constant governing Mg$^{2+}$ binding to R-PPase and its variants ($K_{M,mers}$) were estimated from the protection afforded by this cation against enzyme inactivation by mersalyl (Fig. 5). The dependences shown in Fig. 5 were simultaneously fit to Equations 5 and 6. The parameters $K_{E,mers}$ and $K_{EM,mers}$ were constrained in these fittings to the values obtained above. The resulting $K_{E,mers}$ values (Table II) indicate that E649D is the only variant in which Mg$^{2+}$ binding affinity is significantly affected (a 2-fold decrease).

**1,1-Diphosphonate Binding**—The binding affinities for two non-hydrolyzable substrate analogues (AMDP and MDP) were estimated by measuring their inhibitory activities on PP, hydrolysis (Fig. 6). The value of the inhibition constant, $K_i$, derived from the data with Equation 7 remained unchanged for
FIG. 5. Mg\(^{2+}\) concentration dependence of wild-type and variant R-PPase inactivation by mersalyl. IMVs were incubated with mersalyl for 10 min (wild-type, E197D, and E202D), 2 min (E649D), or 1 min (E550D) at the indicated Mg\(^{2+}\) concentrations. Other details were as for Fig. 4. Lines are drawn in accordance with Equation 7 using the parameter values specified in Table II.

FIG. 6. The effects of aminomethylenediphosphonate (A) and methylenediphosphonate (B) on the hydrolytic activities of wild-type and variant R-PPases. Lines are drawn in accordance with Equation 7 using the parameter values specified in Table II. The symbols on the curves are as follows: ■, wild-type R-PPase; ○, E197D; △, E202D; ◻, E550D; and □, E649D. Conditions were pH 7.2, 2 mM [Mg\(^{2+}\)] and 50 μM [Mg\(_{2}\)PPi].

both analogues in the D550E variant and increased by a factor of 3–8 in the other Glu → Asp variants (Table II).

The \(K_i\) values for AMDP measured with the Glu → Ala variants E197A, E550A, and E649A were 0.40 ± 0.06, 0.20 ± 0.03, and 0.44 ± 0.09 μM, respectively. Thus, the effects of the alanine substitutions on AMDP binding were similar except in the case of the E550A variant, which displayed a lower affinity for this substrate analogue than did the corresponding E550D variant.

Effects of Asp and Lys Substitutions—Nine aspartates (187, 191, 642, 669, 673, and 677) and three lysines (184, 195, and 646) in the putative cytoplasmic loops were similarly replaced with glutamates and arginines, respectively. These substitutions did not affect the expression of the variant enzymes in E. coli but, except for K195R, inhibited R-PPase activity measured at 2 mM Mg\(^{2+}\) to a level indistinguishable from the background in our system (data not shown). K195R activity was 10% of that of the wild-type enzyme. The drastic decrease in PPase activity following these substitutions signifies that these mutated Asp and Lys residues are indispensable for catalysis but precludes further studies using the kinetic approaches employed for the Glu → Asp variants.

DISCUSSION

In the present study, we examined the roles of conserved Glu\(^{197}\), Glu\(^{202}\), Glu\(^{550}\), and Glu\(^{649}\) in H\(^{+}\)-PPase of R. rubrum by site-directed mutagenesis and heterologous expression in E. coli. These residues, with the exception of Glu\(^{202}\), can be replaced with aspartates or alanines and display retention of at least 20% PPase activity measured at high Mg\(^{2+}\) (2 mM) concentrations. In view of the finding that the E202D variant retained significant activity, whereas the E202A variant activity declined to a level indistinguishable from the background in our system (~5% wild-type R-PPase activity), we propose that the carboxyl at position 202 of R-PPase sequence is indispensable for enzyme function. All variants active in PP, hydrolysis were also capable of PP\(^{+}\)-energized proton translocation with a magnitude of coupling similar to that of wild-type R-PPase. This finding is consistent with the tentative assignment of glutamates in hydrophilic regions to cytoplasmic loops, which implies that these residues are unlikely to be involved in proton transport. Interestingly, substitution of Glu\(^{202}\) (corresponding to R-PPase Glu\(^{197}\)) in the H\(^{+}\)-PPase of the plant V. radiata to aspartate led to complete loss of PP\(^{+}\)-energized proton translocation despite the retention of >30% hydrolytic activity (21). It is unlikely that this residue is related to proton transport in V. radiata H\(^{+}\)-PPase but not R-PPase. The E263D substitution possibly caused partial decoupling in the plant enzyme indirectly, perhaps by disrupting the more fragile overall structure. Indeed, a weak current arising from PP\(^{+}\)-energized proton transport was recently detected in the E263D variant of V. radiata H\(^{+}\)-PPase by patch clamp analysis of giant yeast vacuoles (27). In contrast, our data on the D187E, D669E, D673E, D677E, and K196R R-PPase variants are consistent with the results for V. radiata H\(^{+}\)-PPase reported by Nakanishi et al. (21).

Detailed steady-state kinetic analysis performed at a wide range of Mg\(^{2+}\) concentrations indicated that the common effect of all four Glu → Asp substitutions in R-PPase is reduced catalytic efficiency (signified by \(k_{cat}/K_m\)) as compared with that of wild-type R-PPase at low Mg\(^{2+}\) concentrations (<0.5 mM). In terms of Scheme I, this observation is explained by hydrolysis of PP by the variant enzyme via the EM\(_{PP}\) complex only. In contrast, the wild-type enzyme additionally hydrolyzes PP\(^{+}\) via the EM\(_{PP}\) complex. Thus, in the absence of conserved Glu, two metal ions are insufficient to form a catalytically competent active site structure that is restored upon binding of the third metal ion. Similar effects of active site substitutions were observed previously in soluble PPases. Whereas catalysis by wild-type E. coli and S. cerevisiae PPases proceeds through both three- and four-metal pathways (28, 29), active site variants require the whole complement of four metal ions, but are inactive when only three metal ions bind (30, 31). The similarities between the effects of the substitutions in two non-homologous PPase families imply that conserved Glu residues of R-PPase also form part of an active site.

This suggestion is consistent with the effects of the substitutions on the binding of the non-hydrolyzable substrate analogues AMDP and MDP. The \(K_i\) values reported here for AMDP and MDP are true binding constants, in contrast to the Michaelis constant for substrate, which is generally a complex combination of microscopic rate constants for individual reaction steps. AMDP is a 30-fold more potent inhibitor of H\(^{+}\)-PPase in comparison to MDP, which lacks an aminomethyl group in its structure. However, the effects of the substitutions were similar for AMDP and MDP, suggesting that enzyme interactions with the phosphate moieties of the inhibitors were affected. We thus interpret the reduced affinity of the E197D, E202D, and E649D variants for AMDP and MDP as possible evidence of impaired PP\(^{+}\) binding in these variants. Interestingly, at positions 197 and 649, AMDP binding was similarly impaired by Glu → Asp and Glu → Ala substitutions, suggesting that in these cases aspartates are poor substitutes for glutamates with respect to inhibitor binding. Alternatively, at position 550, AMDP binding was impaired in the Glu → Ala but not the Glu → Asp variant. This finding may be explained by the considerable flexibility in the
vicinity of Glu\textsuperscript{550}, such that the shorter aspartate residue maintains the same interactions as the longer glutamate residue.

The catalytic incompetence of the EM\textsubscript{2}PP complex and its decreased affinity to substrate analogues and, presumably, to substrate in the E197D, E202D, and E649D variants and to Mg\textsuperscript{2+} in the E649D variant indicate that the active site structure is significantly distorted by the substitutions. However, the sources of this distortion are distinct in the different variants. In E550D and E649D, this may be a long-range conformational change as indicated by the increased mersalyl sensitivity that may result from increased accessibility of Cys\textsuperscript{573} or other previously inaccessible Cys residues. Thus, Glu\textsuperscript{550} and Glu\textsuperscript{649} likely function in maintaining the R-PPase conformation. In contrast, Glu\textsuperscript{197} and Glu\textsuperscript{202} may be directly involved in metal cofactor binding. According to Scheme I, the enzyme has a metal-binding site (M1) to form EM and an additional metal-binding site (M2) to bind M\textsubscript{2}PP if one of the two metal ions that come with PP, bridges PP, and the enzyme in the resulting EM\textsubscript{2}PP complex as in the case of soluble PPases (32, 33). Glu\textsuperscript{197} may be part of the M1 site as its substitution markedly increases \( K_{M1} \), but not \( K_{A1} \) and \( K_{A2} \) (Table II), whereas Glu\textsuperscript{202} possibly belongs to both the M1 site and the M2 site because its substitution increases all of these parameters. The contradictory invariance of \( K_{M,anes} \) in the E197D and E202D variants (Table II) may result from the fact that this parameter provides a measurement of overall metal binding, whereas the kinetically determined \( K_{M1} \), \( K_{A1} \), and \( K_{A2} \) parameters evaluate only functional binding. The flexibility of the protein structure allows for efficient metal binding in the variant enzymes, but the bulk of the resulting complex is non-productive because the metal is mispositioned. Notably, the lack of effect of similar substitutions (Asp \rightarrow Glu) of metal ligands on thermodynamically controlled metal binding is well documented in soluble PPases (34, 35).

In summary, all of the conserved glutamates in R-PPase are essential for catalytic efficiency at low Mg\textsuperscript{2+} concentrations, which accounts for their conservation in H\textsuperscript{+}-PPases. In addition, Glu\textsuperscript{197} and Glu\textsuperscript{202} may contribute to substrate binding by controlling metal binding but do not affect the overall enzyme structure. On the other hand, Glu\textsuperscript{550} and Glu\textsuperscript{649} may be important for maintaining the catalytically competent conformation of R-PPase.

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