Mechanism of the formation of proton transfer pathways in photosynthetic reaction centers

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In photosynthetic reaction centers from purple bacteria (PbRCs) from *Rhodobacter sphaeroides*, the secondary quinone *Q*₂ accepts two electrons and two protons via electron-coupled proton transfer (PT). Here, we identify PT pathways that proceed toward the *Q*₂ binding site, using a quantum mechanical/molecular mechanical approach. As the first electron is transferred to *Q*₂, the formation of the Grothuss-like pre-PT H-bond network is observed along Asp-L213, Ser-L223, and the distal *Q*₂ carbonyl O site. As the second electron is transferred, the formation of a low-barrier H-bond is observed between His-L190 at Fe and the proximal *Q*₂ carbonyl O site, which facilitates the second PT. As *Q*B₂ leaves PbRC, a chain of water molecules connects protonated *Q*₂L212 and deprotonated His-L190, which serves as a pathway for the His-L190 reprotonation. The findings of the second pathway, which does not involve Glu-L212, and the third pathway, which proceeds from Glu-L212 to His-L190, provide a mechanism for PT commonly used among PbRCs.

Proton-coupled electron transfer | low-barrier hydrogen bond | photosystem II | conformational gating | artificial photosynthesis

Purple bacterial photosynthetic reaction centers (PbRCs) have special pair bacteriochlorophylls (*P*L/PM), accessory bacteriochlorophylls (*B*L/BM), bacteriopheophytins (*H*L/HM), and ubichromes (*U*L/UM) in the heterodimeric L/M protein subunit pair. *P*L and PM form the electronically coupled special pair [P*L*PM]. The electronic excitations of [P*L*PM] leads to the formation of the charge-separated state, [P*L*PM]−[B*L*BM]−, and subsequent electron transfer occurs to *Q*B via H₅ and QA (1–3). *Q*B accepts two electrons via QA and two protons via the proton transfer (PT) pathways, forming Q₂H₂ and leaves the PbRC.

The first and second electron transfers from QA to *Q*B occur with rates *κ*₂₁ (10⁷ s⁻¹) and *κ*₂₂ (10³ s⁻¹), respectively (4). The first PT leads to the protonation of the distal and carbonyl O site of *Q*B (with respect to the nonheme Fe). The proton donor of the distal *Q*B O site is Ser-L223, for which the H-bond donor is His-L190. The first PT leads to *Q*BH₂ formation (8, 22). However, the PT pathway from Glu-L212 to *Q*BH⁻ is unclear (4), because the crystal structure shows that Glu-L212 cannot form an H-bond with the proximal *Q*B O site (Glu-L212...*Q*B = 5.7 Å) (23). PT occurs most efficiently along H-bonds (24). Okamura et al. (4) proposed that the movement of *Q*BH⁻ toward Glu-L212 and the formation of an H-bond might be required for PT. Alternatively, His-L190, which forms an H-bond with the proximal *Q*B O site (His-L190...*Q*B = 2.81 Å) (23), might serve as a proton donor for *Q*BH⁻, as proposed by Wraight (25). However, Wraight also argued that the *p*Kₐ value for the deprotonation of singly protonated His-L190 might be too high even in the presence of the cationic nonheme Fe.

Notably, the crystal structures of PSII and PbRC show large structural similarity (7). His-L190 is conserved as D1-His215 at the nonheme Fe complex in PSII (7). In PSII, D1-His215 can form a low-barrier H-bond with *Q*BH⁻, which facilitates Q₈H₂ formation (8, 26, 27). Low-barrier H-bonds can form when the *p*Kₐ values of the H-bond donor and acceptor moieties are nearly equal (28, 29). The shape of the potential energy curve of a low-barrier H-bond is symmetric, while that of a standard H-bond is asymmetric because *p*Kₐ(donor) > *p*Kₐ(acceptor) (30) (Fig. 1). In addition, Glu-L212 in PbRCs is not conserved as a titratable residue in PSII. These findings for PSII might provide an opportunity to revisit the mechanism of PT toward the proximal *Q*B O site in PbRCs. Here, we report how PT pathways form in response to electron transfer in the PbRC protein environment, by adopting a large-scale quantum mechanical/molecular mechanical (QM/MM) approach based on the PbRC crystal structure (23).

Significance

The crystal structures of photosynthetic reaction centers from purple bacteria (PbRCs) and photosystem II show large structural similarity. However, the proposed mechanisms of proton transfer toward the terminal electron acceptor quinone (*Q*B) are not consistent. In particular, not His-L190, which is a H-bond partner of *Q*B, but rather Glu-L212, which is ~6 Å away from *Q*B, was assumed to be the direct proton donor for *Q*B. We demonstrate that the H-bond between His-L190 and *Q*B is a low-barrier H-bond, which facilitates proton transfer from singly protonated His-L190 to *Q*B. Furthermore, Glu-L212 is not a direct H-bond donor for *Q*B. However, it facilitates proton transfer toward deprotonated His-L190 via water molecules after *Q*BH₂ forms and leaves the PbRC.

Author contributions: H.I. designed research; Y.S., K.S., and H.I. performed research; Y.S., K.S., and H.I. analyzed data; and H.I. wrote the paper.

The authors declare no competing interest.

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Results

The PbRC crystal structure shows that the H-bond network proceeds from the distal Q$_B$ carboxyl O$_r$ site via Ser-L223, Asp-L213, a water molecule, and Asp-M17 (23) (i.e., QM region) (Fig. 2). Note that Asp-L210 cannot form an H-bond with the residues in the H-bond network, since it is more than 5.0 Å away from the nearest residue, Asp-M17 (23). When the first electron transfer occurs to Q$_B$, Glu-L212 protonates and the Ser-L223 hydroxyl group, which initially forms an H-bond with Asp-L213, reorients toward Q$_B$ O$_r$− (6, 31, 32). The H-bond distances along the Q$_B$ O$_r$−…Ser-L223…Asp-L213…H$_2$O…Asp-M17 decrease in response to the proton uptake of Glu-L212 and the reorientation of the Ser-L223 hydroxyl group (Table 1). Specifically, the H-bond between the distal Q$_B$ O$_r$ site and Ser-L223 is shortened from 2.65 to 2.55 Å in response to Q$_B$ form (Table 1), which implies that the first PT proceeds toward the distal Q$_B$ O$_r$ site. The potential energy profile indicates that PT is energetically downhill from Ser-L223 to the distal Q$_B$ O$_r$ site in the presence of Q$_B$ T (Fig. 3A).

Asp-M17 has been reported to be involved in the PT pathway (33). When it accepts a proton from the bulk region, the entire H-bond network that proceeds from Asp-M17 via a water molecule, Asp-L213, and Ser-L223 forms the pre-PT H-bond pattern (Fig. 2B), which serves as a downhill pathway (Fig. 3A): the proton at Asp-M17 is ultimately transferred to the distal Q$_B$ O$_r$ site concertedly via the Grotthuss-like mechanism.

The water molecule that connects Asp-M17 and Asp-L213 (W-H267) is present in the 2.01-Å crystal structure reported by Fujiy and colleagues (34) (PDB ID code 3I4D), whereas it is absent in the light-exposed Q$_B$ T structure reported by Stowell et al. (23) at 2.60-Å resolution (PDB ID code 1AIG). It should also be noted that the distribution pattern of water molecules shows that the Asp-L213 and Asp-M17 moieties are highly accessible from the protein bulk surface (Fig. 4A). Thus, Asp-L213…Ser-L223…Q$_B$ is likely to be the essential component for the first PT, as the corresponding H-bond network, D1-His252…D1-Ser264…Q$_B$ is conserved in PSII (8). Consistently, the potential energy profile indicates that the proton at Asp-L213 can be transferred via Ser-L223 to the distal Q$_B$ O$_r$ site along a downhill path even if Asp-M17 is ionized (Fig. 3B).

As the Q$_B$H$^+$ forms (via the first PT and the second electron transfer), the H-bond between His-L190 and the proximal Q$_B$ O$_r$ site is significantly shortened to 2.49 Å (Table 1). The potential energy profile for the H-bond between His-L190 and the proximal Q$_B$ O$_r$ site resembles that for a typical low-barrier H-bond (Fig. 3C), as observed for the H-bond between D1-His215 and the proximal Q$_B$ O$_r$ site in PSII (8, 26, 27). Thus, the second PT can proceed along the barrierless potential, leading to the release of Q$_B$H$_2$ from the PbRC.

Not only the crystal structure (23) but also the calculated distribution pattern of water molecules shows that no H-bond network exists between Glu-L212 and His-L190 in the presence of Q$_B$ (Fig. 4A). In contrast, the Q$_B$-lacking crystal structure shows that water molecules form an H-bond network that connects Glu-L212 and His-L190 (e.g., PDB ID code 1L9B) (35) (Fig. 4B), which may also be the case with the Q$_B$H$_2$-released PbRC. QM/MM calculations also indicate that an H-bond network forms among protonated Glu-L212, water molecules, and deprotonated His-L190 (Fig. 4C). The potential-energy profile along the H-bond network suggests that pK$_A$(Glu-L212) ≈ pK$_A$(His-L190) (Fig. 5B).

The distribution pattern of water molecules suggests that there are more water molecules than are identified at the Q$_B$ binding moiety in the crystal structure (Fig. 4B). As the addition of a water molecule to the H-bond network (Fig. 5C) decreases the energy barrier for the PT (Fig. 5D), the existence of water molecules, which are not visible in the reported crystal structure, may facilitate PT from Glu-L212 to His-L190 in the Q$_B$H$_2$-released PbRC.

Discussion

The initial protonation of Q$_B$ occurs at the distal O$_r$ site, as the H-bond between Ser-L223 and the distal Q$_B$ O$_r$ site is specifically shortened in response to the formation of Q$_B$ H$^+$ (Fig. 2A and B). The present results indicate that Ser-L223, Asp-L213, a water molecule, and Asp-M17 form a Grotthuss-like PT pathway toward the distal Q$_B$ O$_r$ site (Figs. 2A and 3A). Among them, protonated Asp-L213 (5, 22, 31, 36–38) [pK$_A$(Asp-L213) = 8.9 (38)] and Ser-L223 are conserved as D1-His252 and D1-Ser264 in PSII, respectively (39). Because D1-His252 and D1-Ser264 serve as a PT pathway toward the distal Q$_B$ O$_r$ site in PSII (8), Ser-L223 and Asp-L213 are the primary components for PT toward the distal Q$_B$ O$_r$ site in the PbRC. Consistently, the D(L213)N (9) and S(L223)A (10, 11) mutations affected k$_{AB}$(2) by factors of 6,000 and 400, respectively, whereas the D(M17)N mutation affected k$_{AB}$(2) only by a factor of 2 (4). The extension of the H-bond network [e.g., Asp-M17, Asp-L210 (33, 40), Asp-H124, His-H126, His-H128 (12, 41), and water molecules] may increase the size of the proton entry point. It seems plausible that the proton can enter either directly from Asp-L213 or the extension of the H-bond network.

Glu-L212 was assumed to serve as a proton donor to the proximal Q$_B$ O$_r$ site (e.g., refs. 4, 42, and 43). However, Glu-L212 cannot form an H-bond with Q$_B$ and is unlikely to serve as a direct proton donor for Q$_B$, because PT occurs most efficiently along H-bonds (24). The findings of His-L190 forming a low-barrier H-bond with the proximal Q$_B$ O$_r$ site and being able to serve as a direct proton donor for Q$_B$H$^+$ (Fig. 3C) suggest that pK$_A$(His-L190) ≈ pK$_A$(Q$_B$H$_2$/Q$_B$H$^+$). The involvement of His-L190 in Q$_B$H$_2$ formation is consistent with the mechanism proposed by Wraight (25) and the mechanism suggested for PSII (8, 26, 27). In PSII, QM/MM calculations showed that D1-His215 forms a low barrier H-bond with Q$_B$H$^+$, facilitating PT (8, 26).
Recent Fourier transform infrared (FTIR) studies suggested that D1-His215 can serve as a proton donor to anionic QBH$^-$, as D1-His215 releases the proton upon the oxidation of the nonheme Fe complex (27). His-L190 is conserved as D1-His215 at the nonheme Fe cluster in PSII, whereas Glu-L212 is not conserved. These features suggest that His-L190 is the proton donor for the proximal O site of QB in the PbRC.

The QB binding pocket has a large cavity. The QBH$_2$ channel is oriented toward the protein bulk surface in the transmembrane region (44) (Fig. 4).

### Table 1. H-bond distances near QB (Å)

| PT pattern near QB | L190–QB | QB–L223 | L223–L213 | L213–H$_2$O | H$_2$O–M17 |
|--------------------|---------|---------|-----------|-------------|-----------|
| PDB                |         |         |           |             |           |
| 1AIG*              | 2.81    | 3.21    | 3.07      | —           | —         |
| 3I4D†              | 2.57    | 2.60    | 2.51      | 2.73        | 3.53      |
| QM/MM              |         |         |           |             |           |
| [deprotonated Glu-L212]‡ |   |         |           |             |           |
| QB L223–L213§      | 2.67    | 2.67    | 2.79      | 3.39        | 2.68      |
| QB L223–QB$^-$     | 2.74    | 2.64    | 2.70      | 2.75        | 2.57      |
| [protonated Glu-L212]‡ |   |         |           |             |           |
| QB$^+$ pre-PT#     | 2.65    | 2.55    | 2.70      | 2.98        | 2.61      |
| QB$^+$ post-PT#    | 2.70    | 2.52    | 2.62      | 2.60        | 2.85      |
| QBH$^-$ pre-PT#    | 2.49    | 2.57    | 2.65      | 2.62        | 2.85      |
| QBH$_2$ post-PT    | 2.60    | 2.57    | 2.64      | 2.62        | 2.86      |

—, not applicable. Distances of low-barrier H-bonds are in bold.

*Reported as the light-exposed charge-separated QB$^-$ structure (23). The distances in the dark-adapted structure (PDB ID code 1AIJ) (23) are not shown: the QB binding site in the dark-adapted structure is not consistent with that in the light-exposed structure (23), whereas the difference in the QB binding site is not observed in a FTIR difference spectroscopy (67).
†The redox state is not reported (34).
‡Glu-L212 is deprotonated for neutral unprotonated QB and protonated for QB$^-$ in the present calculation.
§Ser-L223 donates an H-bond to Asp-L213 (Fig. 2A).
#See Fig. 2.
from Glu-L212 to His-L190 is not significantly low with respect to PT to the distal (Fig. 3A and B) and proximal Q_B O (Fig. 3C) sites, the PT profile suggests that pK_a(His-L190) ∼ pK_a(Glu-L212) (9.4) (38) (Fig. 5B and D), which would make the protonation of His-L190 by Glu-L212 possible before an unprotonated quinone occupies the Q_B binding site. The H-bond network presented here is likely to represent a minimum component of the reprotonation pathway for ionized His-L190, which is consistent with the mechanism proposed by Wraight (25), specifically a water molecule serving as a direct proton donor to reprotonate ionized His-L190. Cherepanov et al. (45) proposed that the H-bond network that connects Glu-L212 and His-L190 (water bridge) forms in response to the release of Q_BH^2 and prevents the Q_BH^2 to Q_BH^− reversion. The estimated Q_B exchange time is ∼1 ms (46), which is sufficiently long for a few water molecules to approach the Q_B binding site and form the H-bond network: for example, among 103 water molecules in the protein interior near the Mn_4CaO_4 cluster of the PSII crystal structure, 90 water molecules are incorporated into the binding positions in the 50-ns molecular dynamics simulation (47). In addition, some PbRC crystal structures show that the corresponding water molecule also exists near Glu-L212 and His-L190 even when Q_B is only slightly displaced from His-L190 in the binding pocket (e.g., PDB ID code 1AIJ) (23).

The PbRC crystal structure shows that Glu-L212 has an H-bond network that proceeds toward the protein bulk surface [e.g., the Pla and Plb water chains (48)], which may serve as a pathway for the uptake of 0.3 to 0.8 H^+ (17, 18) for ionized Glu-L212.

**Fig. 3.** Potential-energy profiles for the first and second PT processes. (A) PT toward the distal Q_B O site in the presence of (A) protonated Asp-M17 and (B) ionized Asp-M17. The potential-energy profile was calculated, modeling a water molecule at the Asp-M17 and Asp-L213 moieties, which is observed in the crystal structure reported by Fujii and colleagues (34) (PDB ID code 3I4D). (C) PT toward the proximal Q_B O site.

from Glu-L212 to His-L190 is not significantly low with respect to PT to the distal (Fig. 3A and B) and proximal Q_B O (Fig. 3C) sites, the PT profile suggests that pK_a(His-L190) ∼ pK_a(Glu-L212) (9.4) (38) (Fig. 5B and D), which would make the protonation of His-L190 by Glu-L212 possible before an unprotonated quinone occupies the Q_B binding site. The H-bond network presented here is likely to represent a minimum component of the reprotonation pathway for ionized His-L190, which is consistent with the mechanism proposed by Wraight (25), specifically a water molecule serving as a direct proton donor to reprotonate ionized His-L190. Cherepanov et al. (45) proposed that the H-bond network that connects Glu-L212 and His-L190 (water bridge) forms in response to the release of Q_BH^2 and prevents the Q_BH^2 to Q_BH^− reversion. The estimated Q_B exchange time is ∼1 ms (46), which is sufficiently long for a few water molecules to approach the Q_B binding site and form the H-bond network: for example, among 103 water molecules in the protein interior near the Mn_4CaO_4 cluster of the PSII crystal structure, 90 water molecules are incorporated into the binding positions in the 50-ns molecular dynamics simulation (47). In addition, some PbRC crystal structures show that the corresponding water molecule also exists near Glu-L212 and His-L190 even when Q_B is only slightly displaced from His-L190 in the binding pocket (e.g., PDB ID code 1AIJ) (23).

The PbRC crystal structure shows that Glu-L212 has an H-bond network that proceeds toward the protein bulk surface [e.g., the Pla and Plb water chains (48)], which may serve as a pathway for the uptake of 0.3 to 0.8 H^+ (17, 18) for ionized Glu-L212.

**Fig. 3.** Potential-energy profiles for the first and second PT processes. (A) PT toward the distal Q_B O site in the presence of (A) protonated Asp-M17 and (B) ionized Asp-M17. The potential-energy profile was calculated, modeling a water molecule at the Asp-M17 and Asp-L213 moieties, which is observed in the crystal structure reported by Fujii and colleagues (34) (PDB ID code 3I4D). (C) PT toward the proximal Q_B O site.

from Glu-L212 to His-L190 is not significantly low with respect to PT to the distal (Fig. 3A and B) and proximal Q_B O (Fig. 3C) sites, the PT profile suggests that pK_a(His-L190) ∼ pK_a(Glu-L212) (9.4) (38) (Fig. 5B and D), which would make the protonation of His-L190 by Glu-L212 possible before an unprotonated quinone occupies the Q_B binding site. The H-bond network presented here is likely to represent a minimum component of the reprotonation pathway for ionized His-L190, which is consistent with the mechanism proposed by Wraight (25), specifically a water molecule serving as a direct proton donor to reprotonate ionized His-L190. Cherepanov et al. (45) proposed that the H-bond network that connects Glu-L212 and His-L190 (water bridge) forms in response to the release of Q_BH^2 and prevents the Q_BH^2 to Q_BH^− reversion. The estimated Q_B exchange time is ∼1 ms (46), which is sufficiently long for a few water molecules to approach the Q_B binding site and form the H-bond network: for example, among 103 water molecules in the protein interior near the Mn_4CaO_4 cluster of the PSII crystal structure, 90 water molecules are incorporated into the binding positions in the 50-ns molecular dynamics simulation (47). In addition, some PbRC crystal structures show that the corresponding water molecule also exists near Glu-L212 and His-L190 even when Q_B is only slightly displaced from His-L190 in the binding pocket (e.g., PDB ID code 1AIJ) (23).

The PbRC crystal structure shows that Glu-L212 has an H-bond network that proceeds toward the protein bulk surface [e.g., the Pla and Plb water chains (48)], which may serve as a pathway for the uptake of 0.3 to 0.8 H^+ (17, 18) for ionized Glu-L212.

**Fig. 3.** Potential-energy profiles for the first and second PT processes. (A) PT toward the distal Q_B O site in the presence of (A) protonated Asp-M17 and (B) ionized Asp-M17. The potential-energy profile was calculated, modeling a water molecule at the Asp-M17 and Asp-L213 moieties, which is observed in the crystal structure reported by Fujii and colleagues (34) (PDB ID code 3I4D). (C) PT toward the proximal Q_B O site.

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The PbRC crystal structure shows that Glu-L212 has an H-bond network that proceeds toward the protein bulk surface [e.g., the Pla and Plb water chains (48)], which may serve as a pathway for the uptake of 0.3 to 0.8 H^+ (17, 18) for ionized Glu-L212.

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(19–21) upon QA⁺ formation (Fig. 6, panel 1). The protonation of Glu-L212 increases E°m(QB) with respect to E°m(QA), leading to an energetically downhill electron transfer to QA (22). As QA⁺ forms, a nearby OH group of Ser-L223 is oriented away from Asp-L213 toward the distal QB O site, further increasing E°m(QB) (6), transforming the post-PT (Fig. 6, panel 4) into pre-PT patterns (Fig. 6, panel 1), and facilitating QAH⁺ formation (6) (Fig. 6, panel 2). Thus, Glu-L212 plays a role in facilitating the first electron transfer to QA, donating a proton to ionized His-L190 in the absence of QA (Fig. 6, panel 4), and eventually providing the second proton for transfer to QAH⁺ via His-L190 (Fig. 6, panel 3).

Remarkably, Glu-L212 is not conserved in PSII. The absence of a subunit H-like protein in PSII renders D1-His215 (i.e., Asp-L213 in PbRC) exposed to the protein bulk surface (Fig. 4C), which may explain why PSII has no corresponding protonatable residue. Because subunit H is likely to restrict the access of water molecules to the QB binding pocket in the PbRC, having protonated Glu-L212 in the QB binding pocket could be advantageous for the immediate reprotonation of ionized His-L190 and binding of unprotonated QB at protonated His-L190.

In summary, the pathway to the distal QB O site is a Grotthuss-like H-bond network (e.g., Ser-L223 and Asp-L213), which facilitates PT by transforming the pre-PT to post-PT H-bond patterns (Fig. 2B and C). In contrast, the pathway to the proximal QA O site is a single, low-barrier H-bond between QAH⁺ and His-L190 (Fig. 2D), as observed in PSII (8, 26, 27). His-L190 is the proton donor for QAH⁺ and no movement of QAH⁺ toward Glu-L212 (e.g., ref. 4) is required for the QAH⁺ protonation. However, Glu-L212 is ultimately involved in PT to QAH⁺, because protonated Glu-L212 is likely to serve as a proton donor to ionized His-L190 via water molecules in response to the release of QAH₂ (Fig. 5). Moreover, the proton delivered to Glu-L212 during the initial photo-cycle is delivered to QAH⁺ during the next photo-cycle (Fig. 6). The mechanism presented here can also explain why Glu-L212 is crucial for PT (e.g., refs. 5 and 17–22) irrespective of the absence of an H-bond with QA (see discussions in ref. 4) and how the protonation of QAH⁺ to QAH₂ proceeds via His-L190 (25).

**Methods**

**Coordinates and Atomic Partial Charges.** The atomic coordinates were taken from the X-ray structure of PbRC from *Rhodobacter sphaeroides* (PDB ID code 1A1G) (23). The H atom positions were optimized with CHARMM (49), whereas the heavy atom positions were fixed. During the procedure, all titratable groups (e.g., acidic and basic groups) were ionized. Atomic partial charges of the amino acids were obtained from CHARMM22 (50) parameter set, whereas those of cofactors were obtained from the previous studies (51). Additional counter ions were added to neutralize the entire system in QM/MM calculations.

**Protonation Pattern.** The computation was based on the electrostatic continuum model, solving the linear Poisson–Boltzmann equation with the MEAD program (52). The difference in electrostatic energy between the two protonation states, protonated and deprotonated, in a reference model system was calculated using a known experimentally measured pKₐ value [e.g., 4.0 for Asp (53)]. The difference in the pKₐ value of the protein relative to the reference system was added to the known reference pKₐ value. The experimentally measured pKₐ values employed as references were 12.0 for Arg, 4.0 for Asp, 9.5 for Cys, 4.4 for Glu, 10.4 for Lys, 9.6 for Tyr (53), and 7.0 and 6.6 for the Nᵣ and Nᵦ atoms of His, respectively (54–56). All other titratable sites were fully equilibrated to the protonation state of the target site during titration. The dielectric constants were set to 4 inside the protein.

![Fig. 5. (A) H-bond network that connects protonated Glu-L212 and ionized His-L190 in the absence of QA and (B) the potential-energy profile. The potential-energy profile was calculated adding four water molecules (*: L1039, L1049, L1057, and M1067), which are visible in the QA-free crystal structure (PDB ID code 1L9B) (35). (C) Addition of a water molecule (pink circle) to the H-bond network and (D) the potential-energy profile.](https://doi.org/10.1073/pnas.2103203118)
and 80 for water. All computations were performed at 300 K, pH 7.0, and with an ionic strength of 100 mM. The linear Poisson–Boltzmann equation was solved using a three-step grid-focusing procedure at resolutions of 2.5, 1.0, and 0.3 Å. The ensemble of the protonation patterns was sampled by the Monte Carlo method with the Karlsberg program (57). The Monte Carlo sampling yielded the probabilities [protonated] and [deprotonated] of the two protonation states of the molecule.

**QM/MM Calculations.** The unrestricted density functional theory method was employed with the B3LYP functional and LACVP* basis sets, using the QSite (58) program. In the QM region, all the atomic coordinates were fully relaxed. In the MM region, the positions of H atoms were optimized using the OPLS2005 force field, while the positions of heavy atoms were fixed. The initial-guess wavefunctions were obtained using the ligand field theory (59) implemented in the QSite program. To analyze the potential-energy profiles for PT to Qb, the QM region was defined as the nonheme Fe complex (Fe, the side-chains of His-L190, His-L230, His-M219, His-M266, and Glu-M234), the side-chains of Asp-L213 and Ser-L223, and five water molecules (W-L292, L1039, L1049, L1057, and M1067), which are visible in the 3D-RISM with Placevent analysis was consistent with the positions of the water molecules (65).

**Analysis of Water Molecule Distribution in the Protein.** To analyze the distribution of water molecules in the protein environment, we used a three-dimensional reference interaction site model (3D-RISM) with Placevent analysis (60–64). It should be noted that the distribution pattern of water molecules obtained from the 3D-RISM with Placevent analysis was consistent with the positions of the water molecules (65).

**Data Availability.** All study data are included in the article and Dataset S1.

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