Mechanism of methanol oxidation by quinoprotein methanol dehydrogenase

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At neutral pH, oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$ by an *o*-quinone requires general-base catalysis and the reaction is endothermic. The active-site $-\text{CO}_2^-$ groups of Glu-171 and Asp-297 (Glu-171- CO_2^- and Asp-297- CO_2^-) have been considered as the required general base catalysts in the bacterial *o*-quinoprotein methanol dehydrogenase (MDH) reaction. Based on quantum mechanics/molecular mechanics (QM/MM) calculations, the free energy for MeOH reduction of *o*-PQQ when MeOH is hydrogen bonded to Glu-171- CO_2^- and the crystal water (Wat1) is hydrogen bonded to Asp-297- CO_2^- is $\Delta G^\ddagger = 11.7$ kcal/mol, which is comparable with the experimental value of 8.5 kcal/mol. The calculated $\Delta G^\ddagger$ when MeOH is hydrogen bonded to Asp-297- CO_2^- is >50 kcal/mol. The Asp-297- CO_2^- ···Wat1 complex is very stable. Molecular dynamics (MD) simulations on MDH·PQQ·Wat1 complex in TIP3P water for 5 ns does not result in interchange of Asp-297- CO_2^- bound Wat1 for a solvent water. Starting with Wat1 removed and MeOH hydrogen bonded to Asp-297- CO_2^- , we find that MeOH returns to be hydrogen bonded to Glu-171- CO_2^- and Asp-297- CO_2^- coordinates to Ca^{2+} during 3 ns simulation. The Asp-297- CO_2^- ···Wat1 of reactant complex does play a crucial role in catalysis. By QM/MM calculation $\Delta G^\ddagger = 1.1$ kcal/mol for Asp-297- CO_2^- general-base catalysis of Wat1 hydration of the immediate $\text{CH}_2=\text{O}$ product $\rightarrow \text{CH}_2(\text{OH})_2$. By this means, the endothermic oxidation-reduction reaction is pulled such that the overall conversion of MeOH to $\text{CH}_2(\text{OH})_2$ is exothermic.

hydride transfer | molecular dynamics | pyrroloquinoline quinone

We have had a long standing interest in the mechanism of *o*-quinone cofactor and model reactions (for examples, see refs. 1–4). More recently, our attention has been directed to the employment of computational methods, particularly in the mechanism of the oxidation of methanol and glucose by the appropriate quinoprotein enzymes (5–11).

The present study concerns bacterial quinoprotein methanol dehydrogenase (MDH) catalysis of the oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$ by the pyrroloquinoline quinone (PQQ) cofactor. MDH is a heterotetramer with two heavy and two light subunits. The catalytic chemistry is located in the heavy subunits. The crystal structure (PDB code 1G72; ref. 10) of the reduced PQQ bound to MDH presents Wat1 hydrogen bonding to the $-\text{CO}_2^-$ group of Asp-297 (Asp-297- CO_2^-) and $-\text{CO}_2^-$ group of Glu-171 (Glu-171- CO_2^-) associated with Ca^{2+} . Attempts to crystallize MDH with MeOH substrate at the active site have not yet been successful (10, 12, 13). The hydride transfer mechanism was shown to be correct by x-ray crystallography (10).

During 3-ns molecular dynamics (MD) simulations (6, 7, 9) with MeOH at the active site, it was found that MeOH became hydrogen bonded to Glu-171- CO_2^- , with the C–H of MeOH adjacent to the quinone C5 of PQQ and Wat1 hydrogen bonded to Asp-297- CO_2^- . This MD derived structure is not in accord with the following: (i) the suggestion that Asp-297- CO_2^- serves as the general-base catalyst (14–17); and (ii) that the oxidation takes place, not by hydride transfer, but by an addition elimination reaction (18, 19) (Scheme 1, in which the general base may be Glu-171- CO_2^- or Asp-297- CO_2^-). The MD studies (6, 7, 9) showed that Asp-297- CO_2^- is not in position to act as a general-base catalyst for the oxidation of MeOH by MDH. The mech-

anism of Scheme 2 was proposed. In the present study, we describe the roles played by Glu-171- CO_2^- and Asp-297- CO_2^- in the overall enzymatic reaction: $\text{CH}_3\text{OH} + o\text{-PQQ} + \text{H}_2\text{O} \rightarrow (\text{HO})_2\text{CH}_2 + \text{PQQH}_2$.

Results and Discussions

Evidence Against Asp-297- CO_2^- Being the General Base in the Oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$. In the 3-ns MD simulations (6, 7, 9) of MDH·PQQ·MeOH·Wat1, Wat1 hydrogen bonded to Asp-297- CO_2^- did not exchange with a solvent water, nor exchange position with MeOH hydrogen bonded to Glu-171- CO_2^- . MD simulations, starting with Wat1 hydrogen bonded to Glu-171- CO_2^- and MeOH hydrogen bonded to Asp-297- CO_2^- , show that Wat1 and MeOH exchange hydrogen bonding positions after tens of ps. Also, MD simulations without the presence of MeOH have been carried out to determine whether the Wat1 hydrogen bonded to Asp-297- CO_2^- exchanges with the solvent water. During 0–5 ns, there was no exchange with Wat1 and the distances of O(Wat1) to O5(PQQ) and O(Wat1) to OD1(Asp-297) are 3.11 ± 0.69 Å and 3.15 ± 0.78 Å, respectively [see supporting information (SI) Table 1 and SI Fig. 7]. Thus, the Asp-297- CO_2^- ···Wat1 structure is very stable.

Starting without the presence of Wat1 in the active site and MeOH hydrogen bonded to Asp-297- CO_2^- , as suggested by electron-nuclear double resonance (ENDOR) studies (17), we found that MeOH moves to hydrogen bond to Glu-171- CO_2^- after ≈ 2.2 ns simulation whereas Asp-297- CO_2^- becomes coordinated to Ca^{2+} after ≈ 1.8 ns time (Fig. 1). On the basis of these MD simulation studies, we conclude that the very stable Asp-297- CO_2^- ···Wat1 complex has some role in the enzymatic reaction.

During 3 ns MD simulations of MDH·PQQ·MeOH complex, the structures, in which Asp-297- CO_2^- is hydrogen bonded to MeOH and C–H of MeOH is adjacent to C5 of PQQ, are present in 0.2% of the simulation. It can be seen from Fig. 24 that the reaction barrier for $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$ oxidation involving Asp-297- CO_2^- as the general-base is >50 kcal/mol. This computation means that the reaction with Asp-297- CO_2^- as the general base could not occur. The very large $\Delta G^\ddagger$ for Asp-297- CO_2^- general base catalysis is related to the distance of >3.7 Å between CB (MeOH) and C5 (PQQ).

Glu-171- CO_2^- General Base Catalyst. The energies of the ground state (MDH·PQQ·MeOH·Wat1, Fig. 3), transition state (TS), and immediate product (MDH·PQQ(C5H) $^-\text{O}=\text{CH}_2$ ·Wat1)

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Abbreviations: QM/MM, quantum mechanics and molecular mechanics; SCC-DFTB, self-consistent-charge density-functional tight-binding; MD, molecular dynamics; TS, transition state; PQQ, pyrroloquinoline quinone; MDH, methanol dehydrogenase; MeOH, methanol; ENDOR, electron-nuclear double resonance.

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Scheme 1.

were determined by two-dimensional QM/MM potential energy surface (Fig. 2*B*). The calculated potential energy barrier of Scheme 2 is $\Delta E^\ddagger = 11.5$ kcal/mol by QM/MM. This calculated free energy barrier is comparable with the calibrated value (11.1 kcal/mol) determined by single point calculation at the B3LYP/6-31+G(d,p)/MM level [B3LYP/6-31+G(d,p) as implemented in Gamess-US (20)]. Thus, the present QM/MM (i.e., SCC-DFTB/MM) is reliable for the present system. Normal mode analysis shows that the corrections to the barrier height of zero point energy [$\Delta(ZPE)^\ddagger$], the contributions of entropy ($-T\Delta S^\ddagger$), and the thermal vibrational energy ($\Delta E_{\text{vib}}^\ddagger$) are -2.7 kcal/mol, 2.6 kcal/mol, and -0.7 kcal/mol, respectively. Thus, the calculated free energy barrier for this oxidation reaction is $\Delta G^\ddagger = \Delta E^\ddagger + \Delta E_{\text{vib}}^\ddagger + \Delta(ZPE)^\ddagger - T\Delta S^\ddagger = 11.5 - 0.7 - 2.7 + 2.6 = 10.7$ kcal/mol (Fig. 4), which is in reasonable agreement with 8.5 kcal/mol determined from the experimental Gibbs energy barrier (21) in the wild-type MDH.

Computational findings based on the present QM/MM computations and MD simulations, as well as our previous MD simulations (6, 7, 9), lead to the conclusion that only Glu-171- CO_2^- could be the general base catalyst for the oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$.

Transition State Involving Glu-171- CO_2^- as General Base in the Oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$. The structure (Fig. 5) of the transition state (TS) in Scheme 2 was determined by adiabatic mapping at the QM/MM level, which is almost the same as that (SI Table 1) determined by the conjugate peak refinement (CPR) (22) method. Normal mode analysis confirms the nature of the TS (Fig. 5) with only one imaginary frequency of $170i$ cm^{-1} . In the TS (Fig. 5), the distances of HG1 (MeOH)-OE1 (Glu-171) and HG1 (MeOH)-OG (MeOH) are 1.01 Å and 1.62 Å, respectively; the bond lengths of CB (MeOH)-HB1 (MeOH) and HB1 (MeOH)-C5 (PQQ) are 1.53 Å and 1.22 Å, respectively. This structure indicates an explosive transition state in which proton and hydride are essentially transferred and MeOH has been almost completely converted to formaldehyde ($\text{O}=\text{CH}_2$). Following the Hammond postulate (23), the free energy of the

immediate product $\text{MDH}\cdot\text{PQQ}(\text{C5H})^-\cdot\text{O}=\text{CH}_2\cdot\text{Wat1}$ is close to that of the TS. At this point, the reaction is very endothermic.

Overall Reaction. The oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$ to provide the $\text{MDH}\cdot\text{PQQ}(\text{C5H})^-\cdot\text{O}=\text{CH}_2\cdot\text{Wat1}$ (Fig. 6) is calculated to be endothermic by $\Delta E^\circ = 7.1$ kcal/mol (Fig. 4). Normal mode analysis shows that the correction of $\Delta(ZPE)^\circ$, $-T\Delta S^\circ$, and $\Delta E_{\text{vib}}^\circ$ are -2.0 kcal/mol, 1.6 kcal/mol, and -0.5 kcal/mol, respectively. Therefore, the formation of $\text{MDH}\cdot\text{PQQ}(\text{C5H})^-\cdot\text{O}=\text{CH}_2\cdot\text{Wat1}$ is calculated to be endothermic by $\Delta G^\circ = \Delta E^\circ + \Delta E_{\text{vib}}^\circ + \Delta(ZPE)^\circ - T\Delta S^\circ = 7.1 - 0.5 - 2.0 + 1.6 = 6.2$ kcal/mol (Fig. 4).

The reaction of $\text{H}_2\text{O} + \text{O}=\text{CH}_2 \rightarrow \text{H}_2\text{C}(\text{OH})_2$ is very facile. We propose that Asp-297-CO_2^- catalyzes Wat1 hydration of the $\text{CH}_2=\text{O}$ immediate product. In the $\text{MDH}\cdot\text{PQQ}(\text{C5H})^-\cdot\text{O}=\text{CH}_2\cdot\text{Wat1}$ structure (Fig. 6), the distance of O (Wat1) from the $\text{O}=\text{CH}_2$ carbon is 3.56 Å, and the angle of approach of O (Wat1) to the carbonyl carbon plane of $\text{O}=\text{CH}_2$ is 81° . This is the required geometry (NAC) (24, 25) for the hydration reaction. QM/MM computations show that the following reaction $\text{Glu-171-CO}_2\text{H}\cdots\text{O}=\text{CH}_2\cdots\text{Wat1}\cdots\text{O}_2\text{C-Asp-297} \rightarrow \text{Glu-171-CO}_2^- +$

Fig. 1. MD simulations. (A) Time-dependent variation of the distances between hydroxyl oxygen of MeOH and the carboxylate oxygen of Glu-171- CO_2^- and Asp-297- CO_2^- , respectively. (B) The coordination of Ca^{2+} with O7A (PQQ) and the carboxylate oxygen (OD1) of Asp-297- CO_2^- , respectively, at the ground state of $\text{MDH}\cdot\text{PQQ}\cdot\text{MeOH}$ when Wat1 is replaced by MeOH.

Fig. 2. Contour plot of the potential energy profiles using SCC-DFTB/MM and two-dimensional reaction coordinates $y = r_{\text{CB}(\text{MeOH})\text{-H}\text{B}1(\text{MeOH})} - r_{\text{H}\text{B}1(\text{MeOH})\text{-C}5(\text{PQQ})}$ and $x = r_{\text{OG}(\text{MeOH})\text{-H}\text{G}1(\text{MeOH})} - r_{\text{H}\text{G}1(\text{MeOH})\text{-O}\text{D}1(\text{Asp-297})}$ (A), and $y = r_{\text{CB}(\text{MeOH})\text{-H}\text{B}1(\text{MeOH})} - r_{\text{H}\text{B}1(\text{MeOH})\text{-C}5(\text{PQQ})}$ and $x = r_{\text{OG}(\text{MeOH})\text{-H}\text{G}1(\text{MeOH})} - r_{\text{H}\text{G}1(\text{MeOH})\text{-O}\text{E}1(\text{Glu-171})}$ (B) involving Asp-297-CO₂⁻ general base in MDH-PQQ-MeOH complex and Glu-171-CO₂⁻ general base catalyst in MDH-PQQ-MeOH-Wat1 complex, respectively. The position of the TS is marked by an asterisk.

HOCH₂OH + HO₂C-Asp-297 is associated with a very small (i.e., 1.1 kcal/mol) free energy barrier (Fig. 4). The hydration reaction is calculated to be exothermic by $\Delta G^\circ = -8.7$ kcal/mol at the OM/MM level (Fig. 4).

Fig. 4. The potential surface at the SCC-DFTB/M level (in kcal/mol) for the oxidation reaction of MeOH catalyzed by MDH (solid line) and the hydration of $\text{O}=\text{CH}_2$ (dotted line).

After the departure of $\text{CH}_2(\text{OH})_2$, rearrangement (Scheme 3) to provide the cofactor hydroquinone is exothermic by >4.1 kcal/mol at the SCCDFTB/MM level. Thus, the overall reaction, $\text{MeOH} + o\text{-quinone} + \text{Wat1} \rightarrow \text{CH}_2(\text{OH})_2 + \text{semiquinone}$, is favorable.

Role of Ca^{2+} Ion in the Oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$ with Glu-171- CO_2^- as the General Base. In the QM/MM ground state structure (Fig. 3), Ca^{2+} is at a distance of 2.45 Å from the quinone carbonyl oxygen O5 of PQQ. The late TS places almost a complete negative charge at C5-O of the cofactor and Glu-171- CO_2^- is essentially protonated. Thus, Ca^{2+} electrostatic stabilization of the TS exceeds that with the ground state. Thus, Ca^{2+} plays a catalytic role as in the oxidation of $\text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O}$. This finding is in accord with Ca^{2+} polarizing O5 of PQQ (11). It is to be expected that the interaction of Ba^{2+} with the developing negative charge at the TS would be greater than that of Ca^{2+} . By experiment, it is found that replacement of Ca^{2+} by Ba^{2+} to provide MDH-PQQ- Ba^{2+} system significantly decreases the $\Delta G^\ddagger$ of activation as compared with Ca^{2+} -MDH (3.4 kcal/mol vs. 8.5 kcal/mol) (21).

Fig. 3. The structure of the active site of MDH:PQQ-MeOH-Wat1 complex at the ground state (A) and the close-up of the reaction region (B) as determined by ABNR energy-minimizing the final structure of our previous 3-ns MD simulations at the SCC-DFTB/MM level.

Fig. 5. The structure of the transition state (TS) (Scheme 2) at the active site (A) and the close-up of the reaction region (B) as determined by adiabatic mapping at the SCC-DFTB/MM level.

Conclusion

In order for MeOH to reduce an *o*-quinone by hydride equivalent transfer at neutral pH, the reaction must be general-base catalyzed ($\text{B:} + \text{HOCH}_2\text{H} + >\text{C5}=\text{O} \rightarrow \text{BH} + \text{O}=\text{CH}_2 + >\text{C5H-O}^-$). The only two possible general bases present are Glu-171-CO₂⁻ and Asp-297-CO₂⁻. The following MD observations support Glu-171-CO₂⁻ as the catalytic general base. In the x-ray structure, Glu-171-CO₂⁻ is electrostatically near the Ca²⁺ whereas Asp-297-CO₂⁻ is hydrogen bonded to Wat1. When a molecule of MeOH was placed in various positions in the active site followed by 3 ns MD simulations, the MeOH was found to be hydrogen bonded to Glu-171-CO₂⁻. During 3 ns MD simulations on MDH·PQQ·MeOH·Wat1, Wat1 is hydrogen bonded to Asp-297-CO₂⁻ and not replaced by MeOH. Indeed, in the absence of MeOH, Wat1 does not exchange with the solvent water (TIP3P model) during 5-ns MD simulations.

From the present QM/MM calculations, the free energy barrier ($\Delta G^\ddagger$) for the reaction in which MeOH is hydrogen bonded to Glu-171-CO₂⁻ is 10.7 kcal/mol compared with the experimental value of 8.5 kcal/mol. The transition state for the Glu-171-CO₂⁻ general base hydride equivalent transfer is late with the proton and hydride almost completely transferred and O=CH₂ almost completely formed. In accord with the Hammond postulate, this first step in the overall reaction is endothermic.

Without the presence of Wat1 at the active site and with MeOH hydrogen bonded to Asp-297-CO₂⁻, as suggested by ENDOR studies of Kay *et al.* (17), the value of $\Delta G^\ddagger$ for MeOH reduction of *o*-quinone is at least 50 kcal/mol. The important function for Asp-297-CO₂⁻ hydrogen bonded to Wat1 is to catalyze the hydration of the O=CH₂ direct product. The potential energy barrier for this reaction is calculated to be 1.1 kcal/mol. Most importantly, the exothermic hydration reaction (Eq. 1B) pulls the endothermic hydride transfer reaction (Eq. 1A), such that *o*-quinone + CH₃OH + Wat1 → hydroquinone + H₂C(OH)₂ is thermodynamically favorable:

Although the experiment by ENDOR supported Asp-297-CO₂⁻ as the general base, it must be pointed out that enzyme structure is different at the lower temperature (−60° to −100°C) as compared with ambient temperature, the decrease in the distances of electrostatic interactions occurs with decrease in temperature (26, 27). In addition, the model calculations including MeOH in place of Wat1 show that the reaction does not occur.

Computational Methods

MD Simulations. Based on the procedures described in our previous study (9), stochastic boundary MD was carried out on

Fig. 6. The structure of the intermediate MDH:PQQ(C5H)⁻O=CH₂-Wat1 at the active site as determined by the SCC-DFTB/MM ABNR energy-minimization method.

Scheme 3.