รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษากลไกปฏิกิริยาออกซิเดชันของน้ำตาลไพแรนโนสในปฏิกิริยาของเอนไซม์ Pyranose 2-oxidase จาก *Trametes multicolor*

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Abstract (Thai/English)

สารตัวกลาง (intermediate) C4a-hydroperoxyflavin เป็นสารที่พบในปฏิกิริยา oxidative half-reaction ของเอนไซม์ในกลุ่ม flavin-dependent monooxygenase ซึ่งเป็นเอนไซม์ที่มี FAD (flavin adenine dinucleotide) เป็น cofactor สาร intermediate ดังกล่าวมีความสำคัญต่อการเติม oxygen atom ให้กับสาร aromatic substrate ในขั้นปฏิกิริยา hydroxylation โดยอาศัย oxygen จากอากาศ ในลักษณะเดียวกันกับเอนไซม์ในกลุ่ม flavoprotein oxidase ซึ่งมี flavin เป็น cofactor เป็นที่เชื่อกันมานานว่าเอนไซม์ในกลุ่ม flavoprotein oxidase แยกจากกลุ่มของเอนไซม์ flavin-dependent monooxygenase ได้ เนื่องจากไม่พบการเกิด intermediate C4a-hydroperoxyflavin ในปฏิกิริยาของกลุ่ม flavoprotein oxidase (รูปที่ 1, หมายเลข 2) แต่จากการศึกษากลไกปฏิกิริยา oxidative half-reaction ของเอนไซม์ pyranose 2-oxidase พบว่าเป็นเอนไซม์ในกลุ่ม flavoprotein oxidase ตัวแรกที่พบการเกิด intermediate C4a-hydroperoxyflavin (Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen P. (2008) Biochemistry 47, 8485-8490)

pyranose 2-oxidase เป็นเอนไซม์ที่เร่งปฏิกิริยาการเกิด oxidation ของ D-glucose ที่ C2 ใน pyranose ring ให้เป็น 2-keto-D-glucose โดย electron จาก D-glucose จะถูก transfer ไปยัง FAD cofactor เรียกปฏิกิริยานี้ว่า reductive half-reaction ในขั้นถัดมา electron จาก FAD cofactor จากถูก transfer ไปยัง oxygen และเปลี่ยนเป็น H2O2 (รูปที่ 2) การศึกษานี้ได้ใช้ solvent kinetic isotope effect (SKIE) เพื่อศึกษากลไกของปฏิกิริยา oxidative half-reaction โดยการทำปฏิกิริยาดังกล่าวใน D2O (deuteroxide) แทนที่ H2O ผลที่ได้พบว่า D2O ไม่มีผลต่อ rate constant ในขั้นการเกิด C4a-hydroperoxyflavin intermediate แต่มีผลต่อเฉพาะในขั้นการสลายตัว(decay) ของ intermediate เท่านั้น และเป็นขั้นตอนเดียวกันกับการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H2O2 ผลของ D2O ดังกล่าวทำให้ rate constant ในการสลาย intermediate ช้าลง เมื่อเทียบกับ rate constant ของการสลาย intermediate ใน H2O เป็น 2.8 □ 0.2 เท่า เมื่อศึกษาโดยการใช้ proton inventory พบว่า rate constant ของการสลาย intermediate ลดลงตามสัดส่วนของ deuterium mole atom fraction (D/H) ในลักษณะ linear dependent บ่งชี้ว่า จำนวน

proton นั้นมีเพียงหนึ่ง proton เท่านั้นที่เกี่ยวข้องในการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H2O2 นอกจากนี้การใช้ deuterium labeled ที่ atom ของ nitrogen ที่ N5 ของ flavin ring ใน enzyme โดยใช้ double-mixing stopped-flow spectrophotometer ด้วยการผสม oxidized enzyme กับ 2-deuterated-D-glucose ในขั้นแรก (first mixing) ซึ่งมีผลทำให้ deuterium atom ที่อยู่ที่ C2-D ของ 2-deuterated-D-glucose ไปอยู่ที่ตำแหน่ง N5 ของ flavin ring เกิดเป็น N5-D ผ่านทาง hydride transfer ของปฏิกิริยา reductive half-reaction จากนั้นในขั้นที่สอง (second mixing) reduced enzyme ซึ่งมี N5-D อยู่ที่ flavin ring จะถูก reoxidized โดยการผสมกับ oxygen buffer เพื่อทำให้เกิด C4a-hydroperoxyflavin intermediate ผลปรากฏว่า isotope effect rate constant ในการสลาย intermediate ช้าลงให้ผลเหมือนกับปฏิกิริยาใน D2O ทำให้สรุปได้ว่ากระบวนการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H2O2 เริ่มเกิดจากการเกิด deprotonation ของ hydrogen atom ที่ N5-H (หรือ deprotonation ที่ N5) และ เป็น proton เดียวกันกับที่ทำให้เกิด elimination ไปเป็น H2O2

C4a-hydroperoxy-flavin is commonly found in the reactions of flavin-dependent monooxygenases, in which it plays a key role as an intermediate that incorporates an oxygen atom into substrates. Although its role in monooxygenase reactions has been well established, only recently has evidence for its involvement in the reactions of flavoprotein oxidases been reported. Previous studies of pyranose 2-oxidase (P2O), an enzyme catalyzing the oxidation of pyranoses using oxygen as an electron acceptor to generate oxidized sugars and hydrogen peroxide (H_2O_2), have shown that C4a-hydroperoxy-flavin forms in P2O reactions before it eliminates H_2O_2 as a product [Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen, P. (2008) *Biochemistry 47*, 8485-8490]. In this report, the solvent kinetic isotope effects (SKIE) on the reaction of reduced P2O with oxygen were investigated using transient kinetics. Our results showed that D2O has a negligible effect on the formation of C4a-hydroperoxy-flavin. The ensuing step of H2O2 elimination from C4a-hydroperoxy-flavin was shown to be modulated by an SKIE of 2.8 $\pm$ 0.2, and a proton inventory analysis of this step indicates a linear plot. These data suggest that a single-proton transfer process causes SKIE at the H_2O_2 elimination step. Double and single-mixing stopped-flow experiments performed in H2O buffer revealed that reduced flavin specifically labeled with deuterium at the flavin N5-position generated kinetic isotope effects similar to those found with experiments performed with the enzyme pre-equilibrated in D2O buffer. This suggests that the proton at the flavin N5 position is responsible for the SKIE and is the proton-in-flight that is transferred during the transition state. The mechanism of H2O2 elimination

from C4a-hydroperoxy-flavin is consistent with a single proton transfer from the flavin N5 to the peroxide leaving group, possibly via the formation of an intra-molecular hydrogen bridge.

Reduction Kinetics of 3-Hydroxybenzoate 6-Hydroxylase from *Rhodoccocus jostii* RHA1

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Abstract (Thai/English)

3-hydroxybenzoate 6-hydroxylase (3HB6H) จากเชื้อแบคทีเรีย *Rhodoccocus jostii* RHA1 เป็นเอนไซม์ในกลุ่มเฟลโวโปรตีน โมโนออกซีจีเนส (flavoprotein monooxygenase) เร่งปฏิกิยาการเติมออกซิเจนในสับสเตรทสารประกอบอะโรมาติก 3-hydroxybenzoate (3HB) ที่ตำแหน่งพารา (*para*-position) ได้สารผลิตภัณฑ์ 2,5-dihydroxybenzoate (DHB) โดยอาศัย NADH (nicotinamide adenine dinucleotide) เป็น external reductant

ในงานวิจัยนี้เป็นการศึกษาจลนพลศาสตร์ (kinetics) และ กลไกการเกิดปฏิกิริยา (reaction mechanism) โดยอาศัย stopped-flow spectrophotometer ผลการศึกษาของปฏิกิริยาของเอนไซม์โดย NADH ในภาวะที่ไม่มีสับสเตรท (3HB) ค่า observed rate constant ของการเกิดรีดักชันแปรผันโดยตรงกับความเข้มข้นของ NADH แสดงว่ากลไกการปฏิกิริยารีดักชันโดย NADH เป็นแบบขั้นเดียวด้วย bimolecular rate constant เท่ากับ 43 $\pm$ 2 $M^{-1}s^{-1}$ ในภาวะที่มีสับสเตรท 3HB ค่า observed rate constant ของการเกิดรีดักชันขึ้นกับความเข้มข้นของ NADH ในลักษณะไฮเปอร์โบลิก (hyperbolic) และมีค่า reduction rate มากขึ้นเป็น 48 $\pm$ 2 s^{-1} เมื่อเทียบกับ observed rate reduction ในภาวะที่ไม่มีสับสเตรทซึ่งเท่ากับ 0.43 s^{-1} ที่ความเข้มข้น NADH เท่ากัน เมื่อวัดค่า standard reduction potential (E^0) ของเอนไซม์ที่มีสับเตรท 3HB และ ไม่มี 3HB เท่ากับ -179 $\pm$ 1 mV และ -175 $\pm$ 2 mV แสดงว่าการเปลี่ยนแปลงของค่า reduction rate มาจากการเปลี่ยนแปลงโครงสร้างของเอนไซม์เนื่องจาก enzyme-substrate complex และเป็นปัจจัยสำคัญมากกว่าการเปลี่ยนแปลงทางเทอร์โมไดนามิกส์

3-Hydroxybenzoate 6-hydroxylase (3HB6H) from *Rhodococcus jostii* RHA1 is an NADH-specific flavoprotein monooxygenase involved in microbial aromatic degradation. The enzyme catalyzes the para-hydroxylation of 3-hydroxybenzoate (3 HB) to 2,5-dihydroxybenzoate (2,5-DHB), the ring-fission fuel of the gentisate pathway. In this study, kinetics of the enzyme-bound flavin reduction by NADH was investigated at pH 8.0 using stopped-flow spectrophotometer, and the data were analyzed comprehensively according to kinetic derivations and simulations. Observed rate constants of free enzyme reduction by NADH under anaerobic conditions were linearly dependent on NADH concentrations, consistent with a one-step irreversible reduction model with a bimolecular rate constant of 43 ± 2 $M^{-1}s^{-1}$. In the presence of 3-HB, observed rate constants for flavin reduction were hyperbolically dependent on NADH concentration and approached a limiting value of 48 ± 2 s-1. At saturation concentrations of NADH (10 mM) and 3-HB (10 mM), the reduction rate constant is ~ 51 s^{-1} whereas without 3-HB, the rate constant is 0.43 s^{-1} at a similar NADH concentration. A similar stimulation of flavin reduction was found for the enzyme-product (2,5-DHB) complex, with a rate constant of 45 ± 2 s^{-1}. The rate enhancement induced by aromatic ligands is not due to a thermodynamic driving force because for the enzyme-substrate complex is -179 ± 1 mV compared to of -175 ± 2 mV for the free enzyme. It is proposed that the reduction mechanism of 3HB6H involves an isomerization of the initial enzyme-ligand complex to a fully activated form before flavin reduction takes place.

บทคัดย่อ

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ชื่อโครงการ:การศึกษากลไกปฏิกิริยาออกซิเดชันของน้ำตาลไพแรนโนสในปฏิกิริยาของเอนไซม์ Pyranose 2-oxidase จาก *Trametes multicolor*

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บทคัดย่อ: สารตัวกลาง (intermediate) C4a-hydroperoxyflavin เป็นสารที่พบในการเร่งปฏิกิริยา oxidative half-reaction ของเอนไซม์ในกลุ่ม flavin-dependent monooxygenase ซึ่งเป็นเอนไซม์ที่มี FAD (flavin adenine dinucleotide) เป็น cofactor สาร intermediate ดังกล่าวมีความสำคัญต่อการเติม oxygen atom ให้แก่สาร aromatic substrate ในขั้นปฏิกิริยา hydroxylation โดยอาศัย oxygen จากอากาศ ในลักษณะเดียวกันกับเอนไซม์ในกลุ่ม flavoprotein oxidase ซึ่งมี FAD เป็น cofactor เช่นเดียวกัน เป็นที่เชื่อกันมานานว่าเอนไซม์ในกลุ่มนี้แยกจากกลุ่มของเอนไซม์ในกลุ่ม flavin-dependent monooxygenase เนื่องจากไม่ปรากฏการเกิด intermediate C4a-hydroperoxyflavin ใน catalytic reaction ในการศึกษากลไกปฏิกิริยาของเอนไซม์ pyranose 2-oxidase พบว่าเป็นเอนไซม์ในกลุ่ม flavoprotein oxidase ตัวแรกที่มีการเกิด intermediate C4a-hydroperoxyflavin ((Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen P. (2008) Biochemistry 47, 8485-8490) pyroanose 2-oxidase เป็นนเอนไซม์ที่เร่งปฏิกิริยาการเกิด oxidation ของ D-glucose ที่ C2 ใน pyroanose ring ให้เป็น 2-keto-D-glucose โดย electron จาก D-glucose จะถูก transfer ไปยัง FAD cofactor เรียกปฏิกิริยานี้ว่า reductive half-reaction ในขั้นถัดมา electron จาก FAD cofactor จากถูก transfer ไปยัง oxygen แลเปลี่ยนเป็น H2O2 ในการศึกษานี้จะใช้ solvent kinetic isotope effect (SKIE) ในการศึกษากลไกปฏิกิริยา oxidative half-reaction โดยการทำปฏิกิริยาดังกล่าวใน D2O (deuteroxide) แทนที่ H_2O จากผลที่ได้พบว่า D_2O ไม่มีผลต่อ rate constant ขั้นการเกิด intermediate C4a-hydroperoxyflavin มีผลต่อเฉพาะในขั้นการสลาย (decay) ตัวของ intermediate เท่านั้น และเป็นขั้นตอนเดียวกันกับการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H_2O_2 ผลของ D_2O ดังกล่าวทำให้ rate

constant ในการสลาย intermediate ช้าลงเมื่อเทียบกับ rate constant ของการสลาย intermediate ใน H_2O เป็น 2.8 $\pm$ 0.2 เท่า เมื่อศึกษาโดยการใช้ proton inventory เพื่อหาจำนวน proton (proton number) ที่เกี่ยวข้องให้เกิด SKIE พบว่า rate constant ของการสลาย intermediate ลดลงตามสัดส่วนของ deuterium mole atom fraction (D/H) ในลักษณะ linear dependent บ่งชี้ว่า จำนวน proton นั้นมีเพียงหนึ่ง proton เท่านั้นที่เกี่ยวข้องในการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H_2O_2 นอกจากนี้การใช้ deuterium labeled ที่ atom ของ nitrogen ที่ N5 ของ flavin ring ใน enzyme โดยใช้ double-mixing stopped-flow spectrophotometer ด้วยการผสม oxidized enzyme กับ 2-deuterated-D-glucose ในขั้นแรก (first mixing) ซึ่งมีผลทำให้ deuterium atom ที่อยู่ที่ C2-D ของ 2-deuterated-D-glucose ไปอยู่ที่ตำแหน่ง N5 ของ flavin ring เกิดเป็น N5-D ผ่านทาง hydride transfer ของปฏิกิริยา reductive half-reaction จากนั้นในขั้นที่สอง (second mixing) reduced enzyme ซึ่งมี N5-D อยู่ที่ flavin ring จะถูก reoxidized โดยการผสมกับ oxygen buffer เพื่อทำให้เกิด C4a-hydroperoxyflavin intermediate ผลปรากฏว่า isotope effect rate constant ในการสลาย intermediate ช้าลงให้ผลเหมือนกับปฏิกิริยาใน D_2O ทำให้สรุปได้ว่า กระบวนการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H_2O_2 เริ่มเกิดจากการสลาย H atom ของ N5-H (หรือ deprotonation ที่ N5) และ เป็น proton เดียวกันกับที่ทำให้เกิด elimination ไปเป็น H_2O_2

คำหลัก : pyranose 2-oxidase, flavoprotein oxidase, flavin, rapid kinetics, solvent isotope effect (SKIE)

Abstract

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Project Title : Probing mechanisms of pyranose oxidation in the reactions of pyranose 2-oxidase from *Trametes multicolor*

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Abstract: C4a-hydroperoxy-flavin is commonly found in the reactions of flavin-dependent monooxygenases, in which it plays a key role as an intermediate that incorporates an oxygen atom into substrates. Although its role in monooxygenase reactions has been well established, only recently has evidence for its involvement in the reactions of flavoprotein oxidases been reported. Previous studies of pyranose 2-oxidase (P2O), an enzyme catalyzing the oxidation of pyranoses using oxygen as an electron acceptor to generate oxidized sugars and hydrogen peroxide (H2O2), have shown that C4a-hydroperoxy-flavin forms in P2O reactions before it eliminates H2O2 as a product [Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen, P. (2008) Biochemistry 47, 8485-8490]. In this report, the solvent kinetic isotope effects (SKIE) on the reaction of reduced P2O with oxygen were investigated using transient kinetics. Our results showed that D2O has a negligible effect on the formation of C4a-hydroperoxy-flavin. The ensuing step of H2O2 elimination from C4a-hydroperoxy-flavin was shown to be modulated by an SKIE of 2.8 ± 0.2, and a proton inventory analysis of this step indicates a linear plot. These data suggest that a single-proton transfer process causes SKIE at the H2O2 elimination step. Double and single-mixing stopped-flow experiments performed in H2O buffer revealed that reduced flavin specifically labeled with deuterium at the flavin N5-position generated kinetic isotope effects similar to those found with experiments performed with the enzyme pre-equilibrated in D2O buffer. This suggests that the proton at the flavin N5 position is responsible for the SKIE and is the proton-in-flight that is transferred during the transition state. The mechanism of H2O2 elimination from C4a-hydroperoxy-flavin is consistent with a single proton transfer from the flavin N5

to the peroxide leaving group, possibly via the formation of an intra-molecular hydrogen bridge.

Keywords : pyranose 2-oxidase, flavoprotein oxidase, flavin, rapid kinetics, solvent isotope effect (SKIE)

เนื้อหางานวิจัยประกอบด้วย

1. Abstract (Thai/English)

สารตัวกลาง (intermediate) C4a-hydroperoxyflavin เป็นสารที่พบในปฏิกิริยา oxidative half-reaction ของเอนไซม์ในกลุ่ม flavin-dependent monooxygenase ซึ่งเป็นเอนไซม์ที่มี FAD (flavin adenine dinucleotide) เป็น cofactor สาร intermediate ดังกล่าวมีความสำคัญต่อการเติม oxygen atom ให้กับสาร aromatic substrate ในขั้นปฏิกิริยา hydroxylation โดยอาศัย oxygen จากอากาศ ในลักษณะเดียวกันกับเอนไซม์ในกลุ่ม flavoprotein oxidase ซึ่งมี flavin เป็น cofactor เป็นที่เชื่อกันมานานว่าเอนไซม์ในกลุ่ม flavoprotein oxidase แยกจากกลุ่มของเอนไซม์ flavin-dependent monooxygenase ได้ เนื่องจากไม่พบการเกิด intermediate C4a-hydroperoxyflavin ในปฏิกิริยาของกลุ่ม flavoprotein oxidase (รูปที่ 1, หมายเลข 2) แต่จากการศึกษากลไกปฏิกิริยา oxidative half-reaction ของเอนไซม์ pyranose 2-oxidase พบว่าเป็นเอนไซม์ในกลุ่ม flavoprotein oxidase ตัวแรกที่พบการเกิด intermediate C4a-hydroperoxyflavin (Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen P. (2008) Biochemistry 47, 8485-8490)

pyranose 2-oxidase เป็นเอนไซม์ที่เร่งปฏิกิริยาการเกิด oxidation ของ D-glucose ที่ C2 ใน pyranose ring ให้เป็น 2-keto-D-glucose โดย electron จาก D-glucose จะถูก transfer ไปยัง FAD cofactor เรียกปฏิกิริยานี้ว่า reductive half-reaction ในขั้นถัดมา electron จาก FAD cofactor จากถูก transfer ไปยัง oxygen และเปลี่ยนเป็น H_2O_2 (รูปที่ 2) การศึกษานี้ได้ใช้ solvent kinetic isotope effect (SKIE) เพื่อศึกษากลไกของปฏิกิริยา oxidative half-reaction โดยการทำปฏิกิริยาดังกล่าวใน D_2O (deuteroxide) แทนที่ H_2O ผลที่ได้พบว่า D_2O ไม่มีผลต่อ rate constant ในขั้นการเกิด C4a-hydroperoxyflavin intermediate แต่มีผลต่อเฉพาะในขั้นการสลายตัว(decay) ของ intermediate เท่านั้น และเป็นขั้นตอนเดียวกันกับการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H_2O_2 ผลของ D_2O ดังกล่าวทำให้ rate constant ในการสลาย intermediate ช้าลง เมื่อเทียบกับ rate constant ของการสลาย intermediate ใน H_2O เป็น 2.8 □ 0.2 เท่า เมื่อศึกษาโดยการใช้ proton inventory พบว่า rate constant ของการสลาย intermediate ลดลงตามสัดส่วนของ deuterium mole atom fraction (D/H) ในลักษณะ linear dependent บ่งชี้ว่า จำนวน proton นั้นมีเพียงหนึ่ง proton เท่านั้นที่เกี่ยวข้องในการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H_2O_2 นอกจากนี้การใช้ deuterium labeled ที่ atom ของ nitrogen ที่ N5 ของ flavin ring ใน enzyme โดยใช้ double-mixing stopped-flow spectrophotometer ด้วยการผสม oxidized enzyme กับ 2-deuterated-D-glucose ในขั้นแรก (first mixing) ซึ่งมีผลทำให้ deuterium atom ที่อยู่ที่ C2-D ของ 2-deuterated-D-glucose ไปอยู่ที่ตำแหน่ง N5 ของ flavin ring เกิดเป็น N5-D ผ่านทาง hydride

transfer ของปฏิกิริยา reductive half-reaction จากนั้นในขั้นที่สอง (second mixing) reduced enzyme ซึ่งมี N5-D อยู่ที่ flavin ring จะถูก reoxidized โดยการผสมกับ oxygen buffer เพื่อทำให้เกิด C4a-hydroperoxyflavin intermediate ผลปรากฏว่า isotope effect rate constant ในการสลาย intermediate ช้าลงให้ผลเหมือนกับปฏิกิริยาใน D2O ทำให้สรุปได้ว่ากระบวนการเกิด elimination ของ hydroperoxy group ของ C4a-hydroperoxyflavin intermediate ไปเป็น H2O2 เริ่มเกิดจากการเกิด deprotonation ของ hydrogen atom ที่ N5-H (หรือ deprotonation ที่ N5) และ เป็น proton เดียวกันกับที่ทำให้เกิด elimination ไปเป็น H2O2

C4a-hydroperoxy-flavin is commonly found in the reactions of flavin-dependent monooxygenases, in which it plays a key role as an intermediate that incorporates an oxygen atom into substrates. Although its role in monooxygenase reactions has been well established, only recently has evidence for its involvement in the reactions of flavoprotein oxidases been reported. Previous studies of pyranose 2-oxidase (P2O), an enzyme catalyzing the oxidation of pyranoses using oxygen as an electron acceptor to generate oxidized sugars and hydrogen peroxide (H_2O_2), have shown that C4a-hydroperoxy-flavin forms in P2O reactions before it eliminates H_2O_2 as a product [Sucharitakul, J., Prongjit, M., Haltrich, D., and Chaiyen, P. (2008) *Biochemistry 47*, 8485-8490]. In this report, the solvent kinetic isotope effects (SKIE) on the reaction of reduced P2O with oxygen were investigated using transient kinetics. Our results showed that D2O has a negligible effect on the formation of C4a-hydroperoxy-flavin. The ensuing step of H2O2 elimination from C4a-hydroperoxy-flavin was shown to be modulated by an SKIE of 2.8 $\pm$ 0.2, and a proton inventory analysis of this step indicates a linear plot. These data suggest that a single-proton transfer process causes SKIE at the H_2O_2 elimination step. Double and single-mixing stopped-flow experiments performed in H2O buffer revealed that reduced flavin specifically labeled with deuterium at the flavin N5-position generated kinetic isotope effects similar to those found with experiments performed with the enzyme pre-equilibrated in D2O buffer. This suggests that the proton at the flavin N5 position is responsible for the SKIE and is the proton-in-flight that is transferred during the transition state. The mechanism of H2O2 elimination from C4a-hydroperoxy-flavin is consistent with a single proton transfer from the flavin N5 to the peroxide leaving group, possibly via the formation of an intra-molecular hydrogen bridge.

2. Executive summary

In conclusion, our results clearly show for the first time that the H_2O_2 elimination from C4a-hydroperoxy-flavin in a flavoprotein oxidase reaction is controlled by a proton transfer from the flavin N5 to the peroxide leaving group. Our data indicate that the mechanism might involve the formation of an intra-molecular H-bridge that facilitates the H_2O_2 elimination process. The findings in this report also provide a framework to explain the mechanism that might be involved in the elimination of H_2O_2 from the C4a-hydroperoxy-flavin in other flavin-dependent oxidases and monooxygenases.

3. Objectives

To investigate the proton transfer involved in the formation and decay of the C4a hydroperoxy-flavin intermediate in the reaction of pyranose 2-oxidase using solvent kinetic isotope effects.

4. Experimental Procedures

Reagents. D-glucose (99.5% purity) and horseradish peroxidase were purchased from Sigma-Aldrich Chemie GMG. Deuterated glucose (2-d-D-glucose), sodium deuteroxide (99% purity) and deuterium oxide (99.9% purity) were purchased from Cambridge Isotope Laboratory (USA). ABTS [2,2′-azino-bis(3)-ethybenzenethiazoline-6-sulfonic acid diammonium salt] was purchased from Sigma-Aldrich (Germany). Wild-type P2O was cloned and expressed without a His6-tag, to avoid the interfering properties of the tag, and prepared as previously described (12). Concentrations of the following compounds were determined using the known absorption coefficients at pH 7.0: $\varepsilon_{403} = 1 \times 10^5$ $M^{-1}cm^{-1}$ for peroxidase and $\varepsilon_{458} = 1.3 \times 10^4$ $M^{-1}cm^{-1}$ for the wild-type enzyme (12). The flavin absorption coefficient assumes one FAD bound per subunit.

Spectroscopic studies. UV$\tilde{}$visible absorbance spectra were recorded using a Hewlett-Packard diode array spectrophotometer (HP8453), a Shimadzu 2501PC spectrophotometer or a Cary 300Bio double-beam spectrophotometer. All spectrophotometers were equipped with temperature controlled cell compartments. Enzyme activities were determined by continuous assays using coupled reactions of horseradish peroxidase and its substrate, ABTS, as previously described (33).

Initial rates were calculated from the increase of absorbance at 420 nm resulting from the oxidation of ABTS by H_2O_2 using the molar absorption coefficient of 4.2×10^4 $M^{-1}cm^{-1}$ (per one molar of D-glucose consumed).

Rapid reaction experiments. Reactions were conducted in 100 mM sodium phosphate buffer (pH 7.0) at 4 ºC, unless otherwise specified. Measurements were obtained using a TgK Scientific Model SF-61DX stopped-flow spectrophotometer in either single-mixing or double-mixing mode, as previously described (14). The optical path length of the observation cell was 1 cm. The stopped-flow apparatus was made anaerobic by flushing the flow system with an anaerobic buffer solution containing 10 mM sodium dithionite in 50 mM sodium phosphate buffer, pH 7.0. The buffer for the sodium dithionite solution was made anaerobic by equilibration with oxygen-free nitrogen (ultra-high purity) that had been passed through an Oxyclear oxygen removal column (Labclear). The anaerobic buffer was allowed to stand in the flow system overnight. The flow unit was then rinsed with the anaerobic buffer prior to performing the experiments. Apparent rate constants (k_{obs}) were calculated from the kinetic traces using exponential fits and the software packages Kinetic Studio (TgK Scientific, Salisbury, UK) and Program A (written at the University of Michigan by Rong Chang, Jung-yen Chiu, Joel Dinverno, and D.P. Ballou).

To study the reactions of the reduced enzyme with oxygen in H2O buffer, an anaerobic enzyme solution was equilibrated in an anaerobic glove box (Belle Technology, Portesham, UK) to maintain the concentration of oxygen at levels below 3 ppm, and the enzyme was then reduced with a solution of 10 mM D-glucose in 100 mM sodium phosphate buffer pH 7.0. While adding the solution of D-glucose, enzyme spectra were recorded using a spectrophotometer inside the glove box to ensure complete reduction. The reduced enzyme solution was placed in a glass tonometer and loaded onto the stopped-flow spectrophotometer.

To study solvent kinetic isotope effects on flavin oxidation, all solutions were exchanged with sodium phosphate buffer made with deuterium oxide as previously described (13). In brief, solid sodium phosphate powder of 1.38 g was dissolved in ~ 30 ml of 99.9% (purity) deuterium oxide, and the resulting solution was equilibrated for 13-15 hours (overnight) inside the anaerobic glove box. The equilibrated solution was then evaporated at 60 C° for 3 hours using a rotary evaporator to obtain H2O-free sodium phosphate powder. The resulting powder was added to 99.9% deuterium oxide and the same process was repeated to ensure that the buffer contained at least 99.9% deuterium oxide. The dried powder was then redissolved in ~ 95 ml of 99.9% deuterium oxide. The buffer pD was adjusted by adding solid sodium deuteroxide into the solution while monitoring pH using a pH meter (pD = pH measured + 0.4) (34). The volume of the resulting buffer was adjusted to 100 ml with D2O. 2-d-D-glucose (0.018 g) was

dissolved in ~ 30 ml of 99.9% deuterium oxide, and the solutions were dried twice as described above. To prepare a 10 mM 2-d-D-glucose stock solution, the dried substrate powder was dissolved in 10 ml of 100 mM sodium phosphate buffer in deuterium oxide. The enzyme in D2O buffer was reduced by 2-d-glucose and the reduced enzyme solution was left overnight (~ 18 hr) prior to the stopped-flow experiment. This was to assure that the enzyme was well equilibrated in D2O and all exchangeable sites of the reduced enzyme were incorporated with deuterium.

The oxidation of reduced P2O by molecular oxygen was monitored using the stopped-flow spectrophotometer by following the absorbance at 395 nm for detection of C(4a)-hydroperoxy flavin and 452 nm for oxidized flavin. Solutions with various concentrations of oxygen were made by bubbling certified oxygen/nitrogen gas mixtures of 20%, 50%, 100% and 100% on ice through syringes for 8 minutes. After mixing, this procedure resulted in oxygen concentrations of 0.13 mM, 0.31 mM, 0.61 mM and 0.96 mM, respectively. Equilibration of a buffer on ice with a 100% oxygen/nitrogen gas mixture enables the buffer solution to contain 1.92 mM oxygen before mixing.

P2O was kept at $\tilde{}$ -80 °C in 100 mM MOPS (morpholinopropane sulfonic acid) buffer pH 7.0 prior to its use in solvent kinetic isotope experiments. The concentration of the thawed enzyme solution was increased by reducing the solution volume to ~ 200 μL using a 15 ml Amicon concentrator device with a membrane cutoff size of 10 kDa. The concentrated enzyme was loaded onto a PD-10 column (GE Health Science) that had been equilibrated with either 100 mM sodium phosphate buffer in D2O, pD 7.0, or 100 mM sodium phosphate in H2O, pH 7.0. D2O or H2O buffer was added to elute the enzyme from the column. The resulting solution of P2O in D2O or H2O buffer (~ 2.5 ml) was retrieved, and D2O or H2O buffer was added to obtain the desired volume and enzyme concentration (~ 0.6 of absorbance or ~ 46 μM).

Rate constants were obtained from plots of kobs versus the concentration of oxygen using Marquardt-Levenberg nonlinear fitting algorithms included in KaleidaGraph version 4.0 (Synergy Software). Simulations were performed by numerical methods using Runge-Kutta algorithms implemented in Berkeley Madonna 8.3 and a time-step of 1×10^{-4} s. The model and methods used for simulations of the P2O oxidative half-reaction have been previously described (12-14).

Proton inventory. Two hundred microliters of the solution of concentrated enzyme were loaded onto a PD-10 column equilibrated with either 100 mM sodium phosphate buffer in D2O, pD 7.0, or 100 mM sodium phosphate in H2O, pH 7.0. D2O or H2O

buffer was added to the column to elute the enzyme. H2O or D2O buffer was added to the equilibrated enzyme solution to obtain a final volume of 9 ml with absorbance at 458 nm ~ 0.6. The enzyme solutions were equilibrated inside the anaerobic glove box for 30 minutes to remove oxygen. A solution of 10 mM D-glucose in 100 mM sodium phosphate buffer, pH 7.0, or 10 mM 2-d-D-glucose in 100 mM sodium phosphate buffer in D2O, pD 7.0, was added to the enzyme solution to generate the reduced enzyme in H2O or D2O buffer, as described in the previous section. Reduced enzyme solutions in various mixtures of H2O and D2O buffers were placed inside tonometers under anaerobic conditions. The mole atom fractions of D/H (n) were prepared and calculated based on the volume ratios of D2O and H2O in a total volume of 3 ml according to methods previously described: n = 0 (100% v/v H2O), n = 0.25 (2.25 ml H2O + 0.75 ml D2O), n = 0.50 (1.5 ml H2O + 2.25 ml D2O), n = 0.74 (0.75 ml H2O + 2.25 ml D2O), n = 1 (100% v/v D2O) (34).

All tonometers containing different mole fractions of deuterium were left overnight (~18 hours) at 4 °C to ensure complete equilibration between the enzyme and deuteroxide buffer. Buffers used in substrate syringes (total volume of 5 ml) were prepared with the same volume ratios of D2O and H2O as in the enzyme tonometers to ensure that the mole atom fractions of D/H were equivalent in both sides of the stopped-flow syringes. The resulting buffers were bubbled with certified oxygen/nitrogen gas mixtures to achieve the desired oxygen concentrations and left overnight (~18 hours) in tightly-closed screw cap tubes inside an anaerobic glovebox.

5. Results

Reaction of reduced P2O with oxygen in D2O. The reactions of reduced P2O with oxygen in this report were conducted in 100 mM sodium phosphate, pH(D) 7.0, rather than 50 mM as in previous reports (12, 14), because increased ionic strength improves the solubility of reduced enzymes in both H2O and D2O buffers (13).

The oxidation of the reduced enzyme (26 μM) by 0.96 mM oxygen (the concentrations after mixing) in H2O (filled-circle traces in Fig. 2A) and D2O (solid traces in Fig. 2A) were monitored at 395 and 458 nm. The kinetic traces in Fig. 2 show that the reactions were biphasic in both D2O and H2O buffers. The first phase (0.002 ~ 0.04 s) was characterized by an increase of absorbance at 395 nm due to the formation of a C4a-hydroperoxy flavin intermediate (Fig. 2A) (12). The second phase (0.04 ~ 0.86 s) consisted of an absorbance decrease at 395 nm and an absorbance increase at 458

nm because of the elimination of H2O2 to form the oxidized enzyme (Fig. 2A and Fig. 3). The observed rate constants (kobs1) for the first phase of the reactions in H2O buffer were dependent on the oxygen concentration and exhibit a slope of 5.6 × 0.2 ± 10^4 M^{-1} s^{-1} (k1app in Table 1) and an intercept of 23 ± 1 s-1 (× symbols in Fig. 4A), similar to the values reported previously (12). A plot of the observed rate constants of the second phase versus the oxygen concentrations shows the apparent rate constant of the H2O2 elimination from C4a-hydroperoxy-flavin (k2app) of 24 ± 2 s-1 (× symbols in Fig. 4B, Table 1). The plots of the first and second observed phases in H2O buffer (Figs. 4A-B) were analyzed according to Equation 1 and Equation 2, respectively (12). The derivations of Equations 1-2 have been demonstrated in reference 12.

$$k_{obs1}^{H_2O} = k_1[O_2] + k_{-1} + k_2 - \frac{k_1 k_2 [O_2]}{k_1[O_2] + k_{-1} + k_2} \quad (1)$$

$$k_{obs2}^{H_2O} = \frac{k_1 k_2 [O_2]}{k_1[O_2] + k_{-1} + k_2} \quad (2)$$

According to Equation 1, the intercept of the plot in Fig. 4A (o symbols) is equal to k-1 + k2. Therefore, kinetic simulations using a two-step consecutive model (Fig. 3), as previously described (12), were used to estimate the k-1 and k2 values and to confirm the validity of the kinetic constants calculated from the graphic method described. Simulations using the parameters listed in Table 1 yielded kinetic traces that agree well with the experimental data (Table 1, data not shown). The analysis based on the kinetic simulations indicates a bimolecular rate constant for the formation of C4a-hydroperoxy-flavin (k1sim) of 6.5 ± 0.3 × 104 M-1s-1, a reverse rate constant (k-1sim) of 2.0 ± 0.1 s-1, and a rate constant for H2O2 elimination from the intermediate (k2sim) of 18 ± 1 s-1 in H2O (Table 1, Fig. 3).

For the reactions in D2O buffer, the enzyme oxidation observed using various oxygen concentrations (0.13, 0.3, 0.61, and 0.96 mM from the lower to upper traces in Fig. 2B) yielded kobs1 for the formation of C4a-hydroperoxy flavin as shown in Fig. 4A (empty circles). A plot of the kobs1 versus the oxygen concentration shows a slope of 5.9 ± 0.3 × 104 M^{-1} s^{-1} and an intercept of ~ 8.5 s^{-1} (empty circles in Fig. 4A, Table 1). A plot of the observed rate constants of the second phase versus the oxygen concentration yielded an apparent rate constant for the H2O2 elimination from C4a-hydroperoxy-flavin (k2app) of 6.9 ± 0.5 s^{-1}.

Equations 1 and 2 and kinetic simulations using the model in Fig. 3, similar to those used for the analysis of the H2O reaction described above, were used for the analysis of the data of the D2O reaction. The analysis yielded a bimolecular rate constant (k1sim) for the formation of C4a-hydroperoxy flavin of 6.5 ± 0.3 × 10^4 M-1s-1 and a reversible rate constant of 2 ± 0.1 s-1 (k-1sim), which are similar to the rate constants of the reaction in H2O buffer (Table 1). The rate constant for H2O2 elimination from C4a-hydroperoxy-flavin (k2sim) in D2O was 6.4 ± 0.3 s-1 (Table 1), showing a solvent kinetic isotope effect (SKIE) of 2.8 (18 s-1 versus 6.4 s-1). Kinetic traces obtained from the simulations using these kinetic parameters agree well with the experimental data (dotted-line traces versus solid line traces, Fig 2B).

The results above indicate that there is a negligible SKIE on k1 (formation of the C(4a)-hydroperoxy-flavin intermediate) because = 1 (6.5/6.5) (Table 1), whereas D2O significantly diminishes the value of k2 (Fig. 4B, Table 1). We determined that the SKIE of was 2.8 (18/6.4) (Table 1). This SKIE value is not an artifact of the ~ 0.49 unit increase of the pKa associated with a reaction when a pH-dependent reaction is performed in D2O (34) because the observed rate constants for H2O2 elimination from C4a-hydroperoxy-flavin at pH 6.5, 7.0, 7.5 are 20.5 ± 0.21 s-1, 20.4 ± 0.27 s-1, 20.2 ± 0.50 s-1, respectively. These results have been demonstrated pH-independent in this pH range (data not shown).

Proton inventory analysis. Reactions of reduced P2O with oxygen at various mole fractions of D/H were carried out to analyze the number of proton sites involved in the elimination of H2O2 from the C4a-hydroperoxy flavin. The proton inventory was analyzed according to the Gross-Butler Equation (34, 35) (Equation 3):

$$k_n = k_o \frac{\prod(1-n+n\phi^T)}{\prod(1-n+n\phi^R)} \qquad (3)$$

where n is the atom fraction of D2O in the solvent mixture, kn is the observed rate constant in a D2O-H2O mixture, k0 is the observed rate constant in H2O, and ϕ_T and ϕ_R are fractionation factors for proton sites at the transition state and the initial state, respectively.

Solutions of the reduced enzyme in buffers with various mole fractions of D/H were mixed with the same buffers containing 0.96 mM oxygen (the concentration after mixing). Kinetic traces monitored at 395 nm are shown in Fig. 5A, in which the traces from the lowest to the highest correspond to the n values of 0, 0.25, 0.50, 0.74, and

1.00, respectively. The reactions in the medium with n = 0 (100% v/v H2O, the bottom trace) and n = 1 (100% v/v D2O, the top trace), and in the mixtures of D2O-H2O, each showed biphasic kinetics; the first phase corresponded to the formation of the C(4a)-hydroperoxy-flavin intermediate and the second phase corresponded to the H2O2 elimination from the C4a-hydroperoxy-flavin, which was similar to the results in Fig. 2 and Table 1. For example, the reaction with n = 0.74 (the second kinetic trace from the top, Fig. 5A) showed that the first phase (0.002-0.04 s) was the intermediate formation with an observed rate constant of 70 s-1, and the second phase (0.04 - 0.5 s) was the H2O2 elimination from C4a-hydroperoxy-flavin with an observed rate constant of 8.2 s-1. A slow third phase (~ 2.4 s-1), which accounted for ~ 10% of the total absorbance change at 458 and 395 nm, was observed in the reaction with n = 0.25. This phase was a small decrease in the absorbance at 395 nm and a small increase in the absorbance at 458 nm. The origin of this slow phase is unknown, though it might be due to the slow equilibration of the newly generated oxidized enzyme, which resulted from the H2O2 elimination, with the outside solvent of a D2O/H2O mixture. Although this small additional kinetic phase may suggest some hidden complexity of the reaction in the D2O/H2O mixture, it did not affect the rate constants of the preceding two major phases used for the evaluation of SKIE nor does it disagree with the kinetic model in Fig 3.

The observed rate constants of the second phase (kobs2) were used for proton inventory analysis to identify the number of protons associated with the elimination of H2O2 from C4a-hydroperoxy-flavin. Plotting the kobs2 versus the mole fraction (n) of D2O (Fig. 5B) reveals a linear relationship, suggesting that only a single proton site contributes to the SKIE on k2. Therefore, the plot was analyzed according to Equation 4, a simplified form of Equation 3 in which only one proton bridge is involved during the transition state.

$$k_n = k_0(1 - n + n\phi^T) \qquad (4)$$

The analysis yielded a fractionation factor ϕ_T of 0.34 ± 0.02 and an SKIE of 2.9. Because the rate constant for the H2O2 elimination in H2O is dependent on the oxygen concentration (12), the proton inventory was also performed at a lower oxygen concentration (0.61 mM after mixing) to verify whether the same result is observed at lower oxygen concentrations. The results obtained using an oxygen concentration of 0.61 mM (ϕ_T = 0.32 ± 0.03) and SKIE (3.1) were quite similar to those observed using 0.96 mM oxygen. These results confirm that both the fractionation factor and SKIE are

independent of the oxygen concentration and are derived from intrinsic properties associated with H2O2 elimination from C4a-hydroperoxy-flavin. Although the plot in Fig. 5B is linear and fits with a one-proton transfer model, another independent piece of evidence was sought to confirm this conclusion. Therefore, experiments (presented in Figs. 6 and 7) were carried out to identify the nature and the identity of the proton involved with the H2O2 elimination process.

Nature of the proton site causing SKIE. The results from the previous section suggest that only one proton site is likely to be responsible for the SKIE on the H2O2 elimination step. The experiments in this section were carried out to determine whether the proton-in-flight can be rapidly exchanged with the outside solvent. A reduced enzyme solution (~ 26 μM in H2O) was mixed with 100 mM sodium phosphate buffer (in either D2O or H2O) equilibrated with an oxygen concentration of 0.96 mM (after mixing). The reactions were monitored at 395 nm to detect the formation and decay of the intermediate. The dotted-line trace (Fig. 6) represents the reaction of reduced enzyme prepared in H2O buffer that was mixed with the oxygenated D2O buffer. This kinetic trace is similar to that of the control reaction, which was mixed with the oxygenated H2O buffer (the solid trace in Fig. 6). For a reference, the trace of the reaction carried out in D2O (from Fig. 2B) is shown as the empty-circle trace (Fig. 6). These results indicate that the reaction kinetics are primarily dependent on the buffer in which the enzyme was prepared in, not the buffer added during the stopped-flow mixing. These data also suggest that the exchange rate of the proton-in-flight takes longer than the period monitored in the stopped-flow experiment (~ 1 s).

In another experiment, the exchange rate of the proton responsible for the SKIE on the H2O2 elimination step was investigated using double-mixing spectrophotometry. A solution of the reduced enzyme (39 μM before mixing) in 100 mM sodium phosphate in D2O, pD = 7.0, was mixed with the same buffer in H2O under anaerobic conditions during the first mixing step of the double-mixing stopped-flow experiments. The second mixing step added an aerobic buffer (100 mM sodium phosphate, pH 7.0 equilibrated with 0.96 mM O2) to the solution resulting from the first mixing step. The effects of various age times between the first and second mixings (0.01 s, 0.1 s, 1 s, 100 s, 200 s, and 300 s) were also examined. A longer age time should allow the exchange process to be more complete, that is, more of the deuterium in the reduced P2O should be replaced by protium. Reactions were monitored at 395 nm to measure the kinetics of C4a-hydroperoxy flavin formation and H2O2 elimination, and at 458 nm to detect flavin oxidation. The observed rate constants for the H2O2 elimination from C4a-hydroperoxy-

flavin at 0.96 mM O2 after mixing (~ 6.1 s^{-1} in D2O versus ~ 17 s^{-1} in H2O, Fig. 4) were used to indicate whether the proton-in-flight causing the SKIE was exchangeable within the age times examined. The results indicated that the rate constant for the H2O2 elimination did not exceed 6.1 s^{-1} (data not shown) for any time point examined. This result suggests that the t1/2 of the exchange rate was larger than 300 s or the rate constant of the exchange was equivalent to or less than 2.3×10^{-3} s^{-1}. These data also indicate that the exchange of the dissociable proton causing the SKIE on the H2O2 elimination step was negligible within a period of 300 s.

Origin of the proton-in-flight causing SKIE on the H2O2 elimination from C4a-hydroperoxy-flavin. To identify the location of the proton-in-flight that controls the process of H2O2 elimination from C4a-hydroperoxy-flavin, a reduced enzyme in H2O buffer that was specifically labeled with deuterium at the flavin N5 was prepared using two stopped-flow mixing methods and tested to determine whether the labeled enzyme generated a similar KIE as the reaction in D2O (Fig. 2). The first method used double-mixing stopped-flow spectrophotometry. A solution of oxidized P2O in H2O buffer (78 μM before mixing) was mixed with an equal volume of a solution of 100 μM 2-d-D-glucose in H2O buffer (before mixing) under anaerobic conditions in the first mixing of the double-mixing stopped-flow experiment to produce a reduced enzyme specifically labeled with deuterium at the flavin N5 by a hydride transfer from 2-d-D-glucose (13, 14). This mixing process was aged for 80 s to allow for complete flavin reduction by 2-d-D-glucose prior to the second mixing with an aerobic buffer (a final oxygen concentration of 0.96 mM). This experiment allowed the N5-D reduced flavin to react with oxygen. All buffers employed in this experiment were prepared in H2O (100 mM sodium phosphate buffer pH 7.0). Therefore, under these conditions, any kinetic isotope effect detected should be due to the N5-D of the reduced P2O.

Kinetic traces detected at 395 nm showed that the kinetic isotope effect resulting from the N5-D of the reduced P2O (filled-circle trace Fig. 7A) was similar to the effect detected when the reaction was carried out in all D2O buffers at the same oxygen concentration (0.96 mM, Fig. 2A) because both traces yielded a similar kobs2 for the H2O2 elimination step. C4a-hydroperoxy-flavin resulting from the reaction of reduced P2O labeled with N5-D in H2O buffer eliminated H2O2 at a rate constant of 7.6 s-1 (t1/2 ~ 0.09 s) (filled-circle trace in Fig. 7A), which is similar to the rate constant of 6.08 s-1 (t1/2 ~ 0.11 s) observed for the reaction in D2O buffer (data in Figs. 2 and 4). A control experiment in which the oxidized enzyme was reduced by a solution of 100 □M

D-glucose (before mixing) using the same double-mixing stopped-flow spectrophotometry setup resulted in an observed rate constant of 19 s^{-1} (solid line trace in Fig. 7A), which is similar to the rate constant observed using H2O buffer (17.1 ± 0.09 s^{-1}, data from Fig. 4 were overlaid for a reference as the dotted-line). The slight difference between the observed rate constant of 7.6 s^{-1} for the H2O2 elimination of N5-D C4a-hydroperoxy-flavin (filled-circle trace in Fig. 7A) versus the value of 6.08 ± 0.05 s^{-1} observed when the reaction was pre-equilibrated and carried out in all D2O buffers may be due to the small loss of N5-D caused by an exchange with the outside H2O buffer. The results in Fig. 7A clearly suggest that the bond-breakage of N5-D during the transition state gives rise to the observed KIE of 2.5 (19 s^{-1}/7.6 s^{-1}) and that this step is the rate-limiting factor for the H2O2 elimination from C4a-hydroperoxy-flavin.

In another experiment, the KIE resulting from the N5-D of the reduced P2O was measured using the stopped-flow single-mixing mode. We used the internal flow paths of the stopped-flow machine to generate the N5-D-labeled reduced P2O under anaerobic conditions. A solution of oxidized P2O (88 μM, before mixing) in H2O buffer was loaded into syringe A of the stopped-flow machine (TgK Scientific) and then was mixed with an equal volume of a solution of 100 μM 2-d-D-glucose in H2O buffer which was loaded into syringe B. Both solutions were manually mixed under anaerobic conditions by pushing the solutions of syringes A and B into syringe D to produce the reduced P2O specifically labeled with deuterium at the flavin N5 (13, 14). This process was allowed to proceed for 80-100 s to achieve complete reduction before the solution in syringe D was mixed with aerobic buffer in syringe C using the single-mixing mode of the stopped-flow machine and monitored at 395 and 458 nm. After mixing and under the stopped-flow measurement, the reaction contained 22 μM of the reduced P2O labeled with N5-D and 0.96 mM O2 in 100 mM sodium phosphate buffer, pH 7.0. The kinetics of the reaction is shown as the empty-circle trace in Fig. 7B, which indicates a rate constant for H2O2 elimination from the intermediate of 7.2 s^{-1}. The kinetic trace of the reduced P2O that was prepared by reduction with 2-d-D-glucose in H2O buffer (empty circle-trace) is similar to the reaction in D2O buffer (Fig. 2, overlaid dotted-line trace in Fig. 7B). When the control reaction for which the same mixing setup was carried out as in the empty circle trace but using D-glucose as a reductant, the observed rate constant of the H2O2 elimination was 17.1 s^{-1} (solid line in Fig. 7B). The results in Fig. 7B indicate that the reduced P2O labeled with N5-D shows a KIE of 2.4

(17.1/7.2) for the H2O2 elimination step, which is similar to the observed KIE of 2.5 from the result in Fig. 7A. The two different mixing setup experiments confirm that the reaction of reduced P2O specifically labeled at N5-D with oxygen in H2O buffer gives rise to an observed KIE that is similar to the observed SKIE of the reduced P2O pre-equilibrated in D2O buffer reacting with 0.96 mM oxygen (SKIE of 2.8 from 17.1/6.08 (data from Fig. 4)). These results suggest that the observed SKIE is mainly a result of the flavin N5-H(D) breakage and that this step mainly controls the H2O2 elimination from C4a-hydroperoxy-flavin.

6. สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

This work has provided the first evidence of solvent kinetic isotope effects (SKIEs) on H2O2 elimination from C4a-hydroperoxy-flavin. The results indicated that for P2O from T. multicolor, an SKIE ( ) of 2.8 ± 0.2 was found for the H2O2 elimination step and that the N5 proton of reduced flavin is the proton-in-flight that causes this SKIE. Another group has examined SKIEs using bacterial luciferase reactions but did not detect SKIEs for the formation and decay of C4a-hydroperoxy-FMN (36).

Our results (Figs. 2 and 4, Table 1) show that D2O had a negligible effect on the bimolecular rate constant for C4a-hydroperoxy-flavin formation (k1 in Fig. 3) and the intermediate decay (k-1). The currently accepted mechanism of the reaction between reduced flavin and oxygen (Fig. 1) predicts that the first step of the reaction involves a one-electron transfer to form a radical pair of flavin semiquinone and superoxide radical, which rapidly collapses to form C4a-hydroperoxy-flavin (1, 2, 15-18). Although a net transfer of one proton is required for the formation of C4a-hydroperoxy-flavin (path 1 in Fig. 1), our results do not identify any solvent isotope effect on this step. These data indicate that the protonation process to form C4a-hydroperoxy-flavin is rapid and is not the rate-limiting step in the formation of the intermediate. We propose that the residue His548, which is located near the flavin ring, may donate a proton for this rapid protonation (29, 30). Because this residue is proposed to be a catalytic base that deprotonates a proton from D-glucose C2-OH during the reductive half-reaction (13), it may be possible for the resulting protonated His 548 to donate a proton for the C4a-hydroperoxy-flavin formation during the oxidative half-reaction.

The proton inventory analysis revealed that the plot of the rate constants for H2O2 elimination from C4a-hydroperoxy-flavin at various D2O fractions (kn) versus n is linear (Fig. 5B), suggesting that a one-proton transfer process is a major factor controlling the rate of this step (35). This result suggests that only one proton-in-flight

was involved during the transition state. The proton inventory analysis also yielded an SKIE of = 2.9 ± 0.2 and a fractionation factor for the transition state (ϕ_T) of 0.34 ± 0.02 (Fig. 5B), implying that deuterium binds to the exchangeable site less tightly than it binds average bulk water molecules (34). A value of ϕ_T in the range of 0.3-0.6 is typically found with proton transfers among O, N, or S atoms (34, 35, 37, 38). Our results in Fig. 7 have confirmed that a single proton bridge contributes to the SKIE.

The results in Fig. 5-7 clearly show that the proton bridge responsible for the SKIE in the H2O2 elimination step is the flavin N5 proton and that the environment surrounding this site is rather enclosed. The first mixing in the stopped-flow experiments (Fig. 7) specifically labeled the flavin N5 position with deuterium by transferring a hydride equivalent from 2-d-D-glucose. Although these experiments were performed in an H2O medium, the kinetics of the reaction were similar to those observed in a D2O medium, in which the enzyme was pre-equilibrated ~ 18 hours in the D2O buffer (Fig. 2). The observed KIE of the H2O2 elimination step (at 0.96 mM O2) for the N5-D-labeled flavin performed in the H2O buffer was 2.5 (19/7.6) from the experiment shown in Fig. 7A that used a double-mixing stopped-flow set-up to generate the N5-D-labeled flavin, and 2.4 (17.1/7.2) from the experiment that manually mixed the oxidized enzyme with 2-d-glucose and used the single-mixing mode of the stopped-flow machine to follow the reaction (Fig. 7B); these results were compared to an observed SKIE of 2.8 (17.1/6.08) (data in Fig. 4) for the reaction performed in D2O at 0.96 mM O2. Therefore, the bond breaking of the flavin N5-H is the key step controlling the overall process of H2O2 elimination from C4a-hydroperoxy-flavin. The exchange rate between deuterium and protium at the flavin N5 site was shown to be quite slow by the fact that the deuterium of the N5-D-labeled flavin remained intact in H2O medium before the second stopped-flow mixing (~ 80-100 s, Fig. 7). Based on the stopped-flow double-mixing experiment with different buffers, the exchange rate constant at the flavin N5 position was estimated to be less than 2.3 × 10-3 s^{-1} (data not shown). Previous work has shown that the exchange rate constant of the N5 proton of free reduced FMN at pH ~ 7 is ~ 242 s^{-1}, which is equivalent to a t1/2 of ~ 0.0029 s at 25 °C (39). These results indicate that the active site environment surrounding the N5-proton in P2O must be enclosed to impede the exchange rate with the outside solvent. During the oxidative half-reaction of P2O, the substrate loop is thought to be in a closed conformation, which is thought to increase the hydrophobicity of the active site environment (12, 29, 30, 32).

Based on the current data, the reaction mechanism underlying the H2O2 elimination from C4a-hydroperoxy-flavin is proposed to involve an intra-molecular H-bridge that facilitates the H2O2 elimination (as shown in Fig. 8). The data reported here clearly show that the N5 proton is responsible for the SKIE, and it is the proton-in-flight during the transition state. The mechanism of H2O2 elimination from C4a-hydroperoxy-flavin is likely to involve a single proton bridge transfer from the flavin N5 position to a peroxide leaving group (Fig. 8). Although an X-ray structure of the C4a-hydroperoxy-flavin transient intermediate of P2O is not available, a potential configuration at the C4a position of the intermediate can be postulated based on the structure of the C4a-flavin-oxygen adduct of choline oxidase, a flavoprotein oxidase in the same superfamily (glucose-methanol-choline oxidoreductases) as P2O (40). According to the choline oxidase adduct structure, the C4a carbon of the flavin adduct assumes an sp3 configuration, similar to that which would be expected if the flavin adduct were a free compound (40). Based on this information, a three-dimensional representation of C4a-hydroperoxy-flavin was generated using ChemDraw 3-D Pro to approximate the distance between the flavin N5-proton and the proximal oxygen atom of the intermediate. Because the configuration of the proximal oxygen is fixed in the structure of C4a-hydroperoxy-flavin, measurements based on this theoretical structure estimate that the distance between the flavin N5 proton and the proximal oxygen is ~ 2.2 Å (data not shown). This distance is too far to allow the flavin N5 proton and proximal oxygen to engage in direct H-bonding interactions to facilitate a one-step proton transfer. Therefore, we propose that the flavin N5-H may initially form an H-bond with the distal oxygen (Fig. 8). When the reaction proceeds, the bond between the flavin C4a and the proximal oxygen becomes more extended because the flavin ring becomes more planar when the flavin N5 and C4a assume their new hybridization and the iminium double-bond starts to form. This should decrease the distance between the flavin N5-H and the proximal oxygen and permit the H-bond interactions (Fig. 8) that facilitate the protonation of the proximal oxygen to generate a stable H2O2 leaving group (Fig. 8).

The intra-molecular H-bridge facilitating the H2O2 elimination mechanism proposed in Fig. 8 is also supported by our previous results showing that mutations of Thr169 (a residue with its side chain [O^{γ}] close enough to interact with the flavin N5) to Ser, Ala or Gly, abolish the formation of the C4a-hydroperoxy-flavin intermediate (Fig. 9A) (31). For the wild-type enzyme, the H-bond interaction between the flavin N5 and the [O^{γ}] of the Thr169 side chain may divert the interaction of the intra-molecular H-bridge that facilitates H2O2 elimination (Fig. 9B). In Thr169 mutants, the optimum H-

bonding interaction between the [O^{γ}] of Thr169 and the flavin N5 is removed, resulting in an environment with lower dielectric constants, which promotes the intra-molecular H-bridge formation and thus facilitates H_2O_2 elimination. Therefore, the abolishment of C4a-hydroperoxy-flavin that is observed in Thr169 mutants may be due to an increased decay of the intermediate (31), that is, an increased rate constant of H_2O_2 elimination. The model in Fig. 8 is also supported by the recent investigation of a flavin-containing monooxygenase showing that an Asn78Ser mutant, in which the site near the C4a/N5 locus is widened and in which the H-bonding interaction between the flavin N5 and NADP+ is altered, voids its ability to form a C4a-(hydro)peroxy-flavin intermediate (41).

The intra-molecular H-bridge proton transfer proposed in Fig. 8 would be supported if the pKa values of the flavin N5-H and the peroxide leaving group were in the same range. The pKa of H_2O_2 is known to be ~ 11.8 (42), whereas a hydroperoxide of a free flavin derivative is ~ 9.2 (16). When bound to the enzyme, the pKa of flavin-C4a-hydroperoxide may be significantly varied. Indeed, it has been reported to be 8.4 for the reaction of cyclohexanone monooxygenase (43) or > 10.0 for the oxygenase component of p-hydroxyphenylacetate 3-hydroxylase (44). The pKa of the N5-H of C4a-hydroperoxy-flavin has not been experimentally measured but has been estimated to be < 17 (45). According to NMR studies, the pKa of the N5-H in free reduced FMN is high (> 20) (42) because it is the second deprotonation of the flavin ring. Merenyi et al., argued that the pKa of the flavin N5-H in the presence of a C4a-hydroperoxy substituent would likely be decreased to below ~ 17 because the flavin ring is neutral (45). In addition, the change of nitrogen hybridization from the amine (pKa < 17) in the C4a-hydroperoxy-flavin (a reactant) to the imine with sp2 hybridization in the oxidized flavin (a product) should help lower the pKa of the flavin N5-H to significantly less than 17. The pKa of C4a=N5-H in the oxidized flavin was estimated to be $\tilde{}$ 8.3 (16). Recently, we have shown that the pKa associated with the H_2O_2 elimination from C4a-hydroperoxy-flavin in the reaction of the oxygenase component of p-hydroxyphenylacetate hydroxylase is > 9.4 (44). Taken together, our data suggest that the pKa values of the flavin N5-H and the C4a-flavin hydroperoxide may not differ significantly and may permit proton transfer via an intra-molecular H-bridge as proposed in Fig. 8.

FIGURE LEGENDS

Fig 1. Reaction of reduced flavin and oxygen. Path 1 is generally observed for flavin-dependent monooxygenases and P2O whereas path 2 is observed for most of flavoprotein oxidases.

Fig 2. Reactions of reduced P2O with oxygen in H_2O and D_2O buffers. (2A) Filled-circle traces are kinetics monitored from mixing reduced P2O (26 μM) in H_2O buffer (100 mM sodium phosphate pH 7.0) with the same buffer containing 0.96 mM oxygen under the stopped-flow spectrophotometer at 4 °C. Solid line traces are kinetics monitored from mixing reduced P2O (26 μM) in D_2O buffer (100 mM sodium phosphate pD 7.0), with the same D_2O buffer under a similar condition as the reaction in H_2O. All concentrations described are for after mixing. Kinetics of the absorbance change at 395 nm shows two phases with the absorbance increase resulted from formation of C4a-hydroperoxy-flavin and the absorbance decrease due to H_2O_2 elimination from the intermediate. Kinetics of the flavin oxidation monitored at 458 nm is coincided with the absorbance decrease of the intermediate at 395 nm. (2B) Solid lines show the reactions shown in 2A, but they were carried out in D_2O buffer at oxygen concentrations of 0.13 mM, 0.31 mM, 0.61 mM and 0.96 mM (shown as the lower to upper traces). Dotted lines are kinetic simulations using rate constants listed in Table 1 and the model in Fig.3 as described in (12).

Fig 3. Reaction mechanism of the reduced P2O with oxygen.

Fig 4. Kinetic analysis of the reactions of reduced P2O with oxygen in D_2O buffer. The observed rate constants from the kinetic traces in Fig. 2 versus the oxygen concentrations (0.13, 0.31, 0.61, 0.96 mM) are shown. Fig. 4A shows a plot of the observed rate constants for the first phase (k_{obs1}) in H_2O (× symbols) and in D_2O (empty circles). The $k_{obs1}^{H_2O}$ from low to high oxygen concentrations are 25.9 ± 0.9 s^{-1}, 29.5 ± 0.3 s^{-1}, 44.8 ± 0.1 s^{-1}, 62.2 ± 0.7 s^{-1}, respectively while the $k_{obs1}^{D_2O}$ are 10.6 ± 0.04 s^{-1}, 19.2 ± 0.24 s^{-1}, 35.5 ± 0.16 s^{-1}, 63.4 ± 0.48 s^{-1}. Fig. 4B shows plots of the observed rate constants of the second phase (k_{obs2}) from the reaction in H_2O (× symbols) and D_2O (empty circles). The $k_{obs2}^{H_2O}$ according to low to high oxygen concentrations are 5.99 ± 0.02 s^{-1}, 12.0 ± 0.07 s^{-1}, 15.9 ± 0.03 s^{-1}, 17.1 ± 0.09 s^{-1}, respectively while the $k_{obs2}^{D_2O}$ are 4.81 ± 0.02 s^{-1}, 6.43 ± 0.08 s^{-1}, 6.63 ± 0.03 s^{-1}, 6.08 ± 0.05 s^{-1}. A vertical line at each data point indicates a standard deviation of the measurement.

Fig 5. The proton inventory analysis for the observed constants of the H_2O_2 elimination from C4a-hydroperoxy flavin. Solutions of the reduced enzyme (26 μM) were mixed with buffer solutions containing 0.96 mM oxygen in the stopped flow spectrophotometer at 4 $^{\circ}$C. Both solutions of the reduced enzyme and the oxygenated buffers were prepared in various mole fractions of D/H and the solutions with the same mole fractions were mixed under the stopped-flow experiments (Experimental Procedure). (5A) The reactions were monitored at 395 nm for measuring kinetics of formation and H_2O_2 elimination of the C4a-hydroperoxy flavin intermediate at various mole fractions of D/H (*n*). Mixtures of 100 mM sodium phosphate pH 7.0 in H_2O and 100 mM sodium phosphate pD 7.0 in D_2O were employed to attain n = 0, 0.25, 0.50, 0.74, 1.00, and their results are shown according to the lower to upper traces. All concentrations are as after mixing conditions. (5B) A plot of the observed rate constants (k_n) for the H_2O_2 elimination from C4a-hydroperoxy, the second phase with a decrease in absorbance at 395 nm (5A), versus the mole fractions of D/H (*n*) shows a linear relationship. The observed rate constants (k_n) according to low to high values of D/H (*n*) are 17.1 $\pm$ 0.09 s^{-1}, 14.0 $\pm$ 0.17 s^{-1}, 11.2 $\pm$ 0.23 s^{-1}, 8.21 $\pm$ 0.04 s^{-1}, 6.12 $\pm$ 0.04 s^{-1}. The data are consistent with a model involving one proton-in-flight during the transition state. A vertical line at each data point indicates a standard deviation of the measurement.

Fig 6. Nature of a proton site causing the solvent kinetic isotope effect. The dotted-line trace was obtained from mixing reduced enzyme (22 μM), which was prepared in H_2O buffer (100 mM sodium phosphate pH 7.0), with 0.96 mM oxygen in D_2O buffer (100 mM sodium phosphate pD 7.0). All concentrations are described as after mixing concentrations. The solid line trace is from mixing reduced enzyme (22 μM), which was prepared in H_2O buffer (100 mM sodium phosphate pH 7.0), with 0.96 mM oxygen in H_2O buffer (100 mM sodium phosphate pH 7.0). The reaction was monitored absorbance change at 395 nm in the stopped-flow spectrophotometer at 4 $^{\circ}$C. The results show that kinetics of the H_2O_2 elimination (the second phase) of both traces are similar, only dependent on the solution in which P2O was prepared and is independent of the buffer in a substrate syringe. For a reference, the kinetic trace of the reaction of reduced enzyme, which was prepared in D_2O buffer, mixing with oxygen (0.96 mM) in D_2O buffer (data from Fig 2.) is shown as the empty circle trace. The empty circle trace was multiplied by a factor of 0.85 to bring the absorbance signal to a similar range as other traces.

Fig 7. Kinetic isotope effects of the reduced enzyme specifically labeled with deuterium at the flavin *N5* position in H_2O buffer. (7A) Oxidation of the reduced enzyme labeled with N5-D was investigated using double-mixing stopped-flow spectrophotometry. During the first mixing, a solution of the oxidized enzyme (78 μM before mixing) was mixed with an equal volume of 100 μM 2-*d*-D-glucose (before mixing) in H_2O buffer under anaerobic condition. The mixture was aged for 80 s after the first mixing, before the reduced enzyme was mixed with 100 mM sodium phosphate buffer containing 0.96 mM oxygen (final concentration) (filled-circle line). Only H_2O buffers were employed in this experiment. The reaction was monitored at 395 nm to determine the rate constant for the H_2O_2 elimination from C4a-hydroperoxy flavin. The control experiment, where a solution of 100 μM D-glucose was used instead of 100 μM 2-*d*-D-glucose, was carried out in a similar fashion, and is shown as a solid line trace. Results show that kinetics of the trace with filled circles (N5-D reduced flavin) is similar to that of the reduced enzyme in D_2O buffer reacting with 0.96 mM oxygen in D_2O buffer (data in Fig. 2) while kinetics of the double-mixing reaction employing D-glucose (solid line) is similar to the reaction carried out in H_2O buffer (dotted-line trace, data from Fig. 2). The dotted-line trace was multiplied by a factor of 0.7 and offset by +0.005 AU to bring the absorbance signal to a similar range as the traces from the double-mixing experiment. (7B) Oxidation of the reduced enzyme labeled with N5-D was carried out using the single-mixing stopped-flow spectrophotometer. Equal volumes of solutions of the oxidized enzyme 88 μM in H_2O buffer (before mixing) in the syringe A and 100 μM 2-*d*-D-glucose in H_2O buffer (before mixing) in the syringe B were manually pushed and mixed in the syringe D under anaerobic conditions. The reaction in the syringe D was allowed to proceed for 80-100 s to obtain the completely reduced P2O labeled with N5-D before the single-mixing stopped-flow experiment took place by mixing the syringe D solution with an aerobic buffer in the syringe C. The final reaction contained 22 μM of the reduced enzyme specifically labeled with N5-D and 0.96 mM oxygen in 100 mM sodium phosphate in H_2O, pH 7.0 (empty-circle trace). The empty-circle trace is similar to the kinetic trace from the reduced enzyme in D_2O buffer reacting with 0.96 mM oxygen in D_2O buffer (Fig. 2). For a reference, the trace from Fig.2B was multiplied by a factor of 0.66, offset by + 0.044 AU, and shown as a dotted-line. The solid line trace was obtained from the control reaction, in which a solution of 100 μM D-glucose was used instead of 100 μM 2-*d*-D-glucose, and the reaction was carried out in a similar fashion to the empty-circle trace. Results of the single-mixing stopped-flow in Fig. 7B are similar to the results of the double-mixing stopped-flow experiment in Fig. 7A. The data in this figure indicate

that the solvent kinetic isotope effect on the H_2O_2 elimination from C4a-hydroperoxy-flavin is mainly contributed by the N5-H bond breaking.

Fig 8. Proposed reaction mechanism for the H_2O_2 elimination from C(4a)-hydroperoxy flavin. Dotted lines represent hydrogen bonds that may form between the flavin N5 proton and the distal and proximal oxygen during the transition state.

Fig 9. P2O active site structure. (A) The active site of P2O in the closed conformation indicates that O^{γ} of Thr169 can make a H-bond interaction with the flavin N5. (29,30) (B) This H-bonding interaction in the wild-type enzyme may divert the intramolecular H-bridge proton transfer, which assists the H_2O_2 elimination from C4a-hydroperoxy-flavin. Therefore, C4a-hydroperoxy-flavin is observed in the wild-type enzyme but not in the mutants of Thr169 (31).

Figure 1

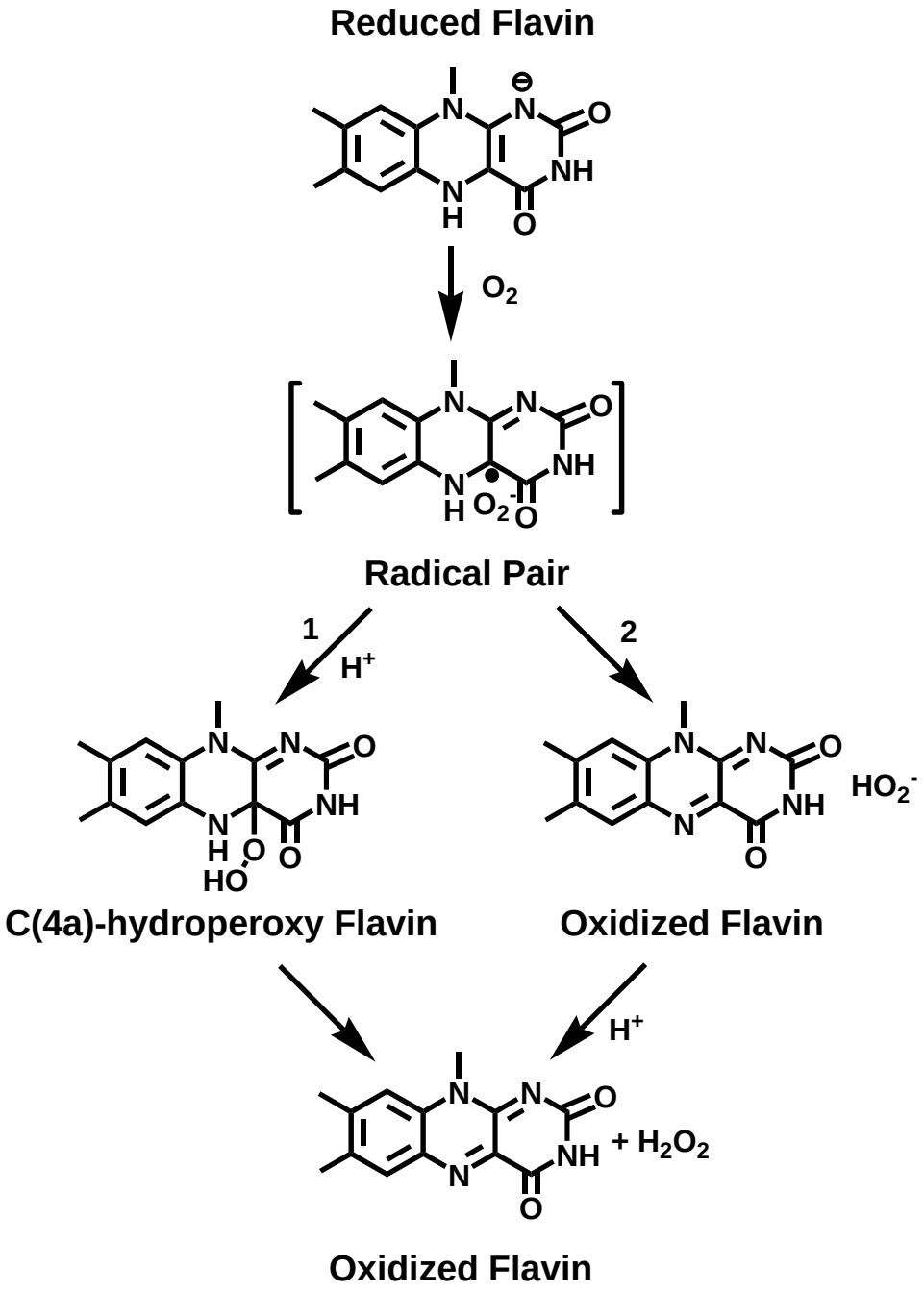

Reduced Flavin
O2
Radical Pair
1
H+
2
HO2-
C(4a)-hydroperoxy Flavin
Oxidized Flavin
H+
+ H2O2
Oxidized Flavin

Figure 2

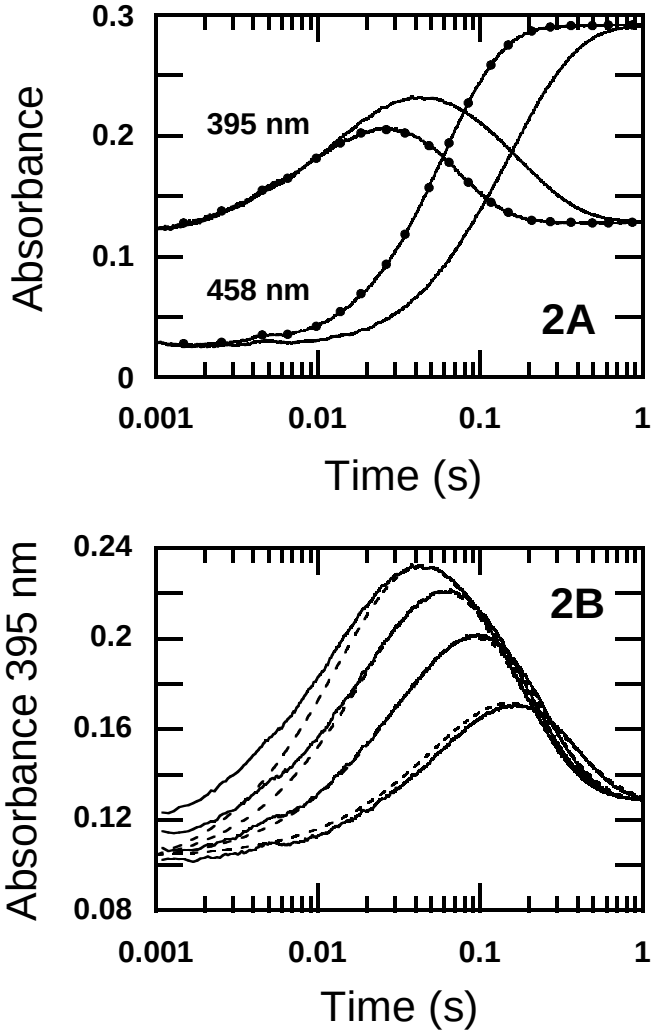
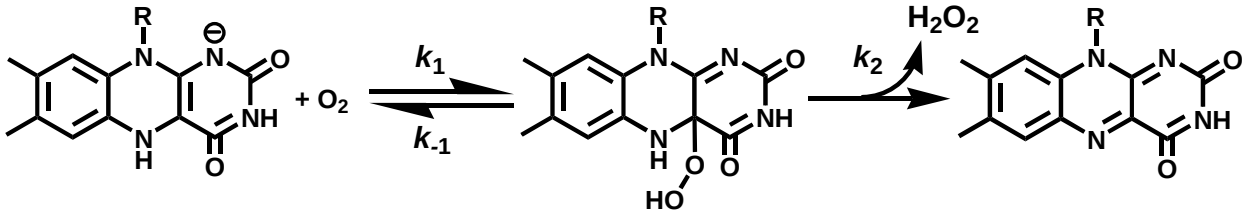

0.3
0.2
0.1
0
Absorbance
395 nm
458 nm
2A
0.001
0.01
0.1
1
Time (s)
0.24
0.2
0.16
0.12
0.08
Absorbance 395 nm
2B
0.001
0.01
0.1
1
Time (s)

Figure 3



Reduced Flavin **C(4a)-hydroperoxy-flavin** **Oxidized Flavin**

Figure 4

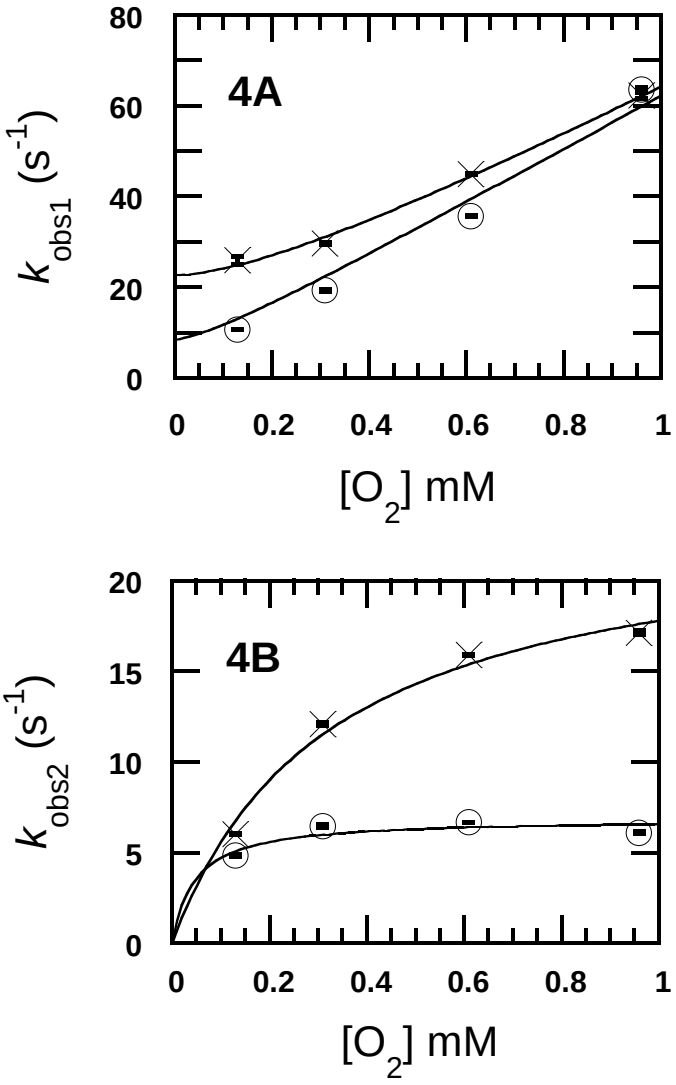

4A
k_{obs1} (s^{-1})
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
[O_2] mM
4B
k_{obs2} (s^{-1})
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
[O_2] mM

Figure 5

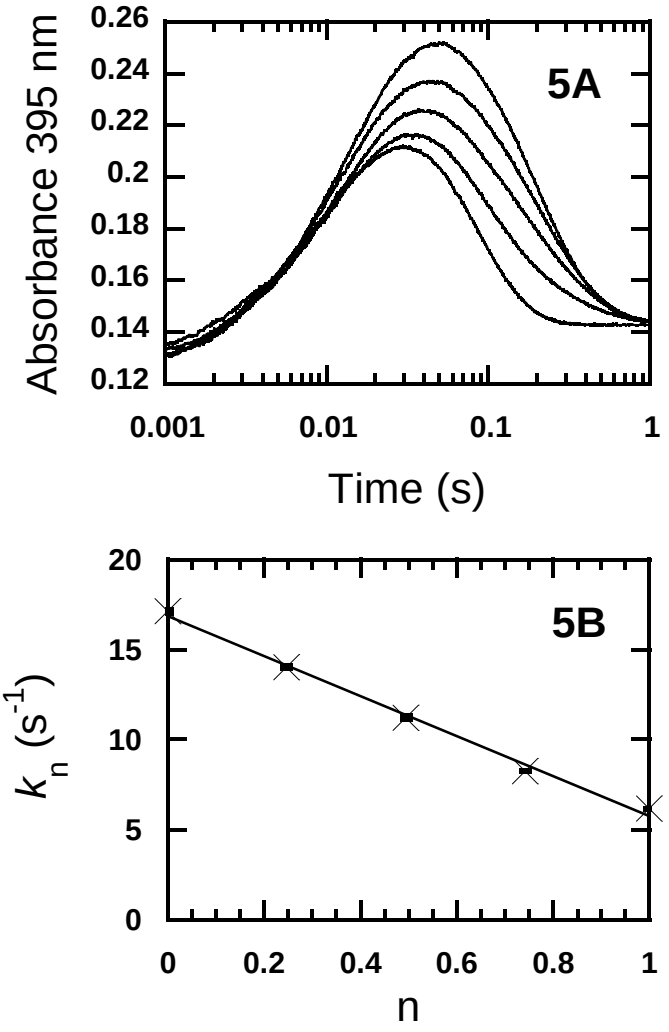

5A
Absorbance 395 nm
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.001
0.01
0.1
1
Time (s)
5B
k_n (s^{-1})
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
n

Figure 6

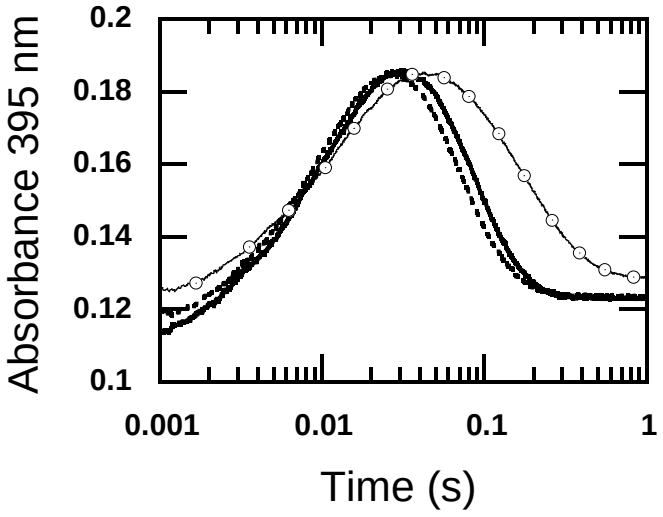


Figure 7

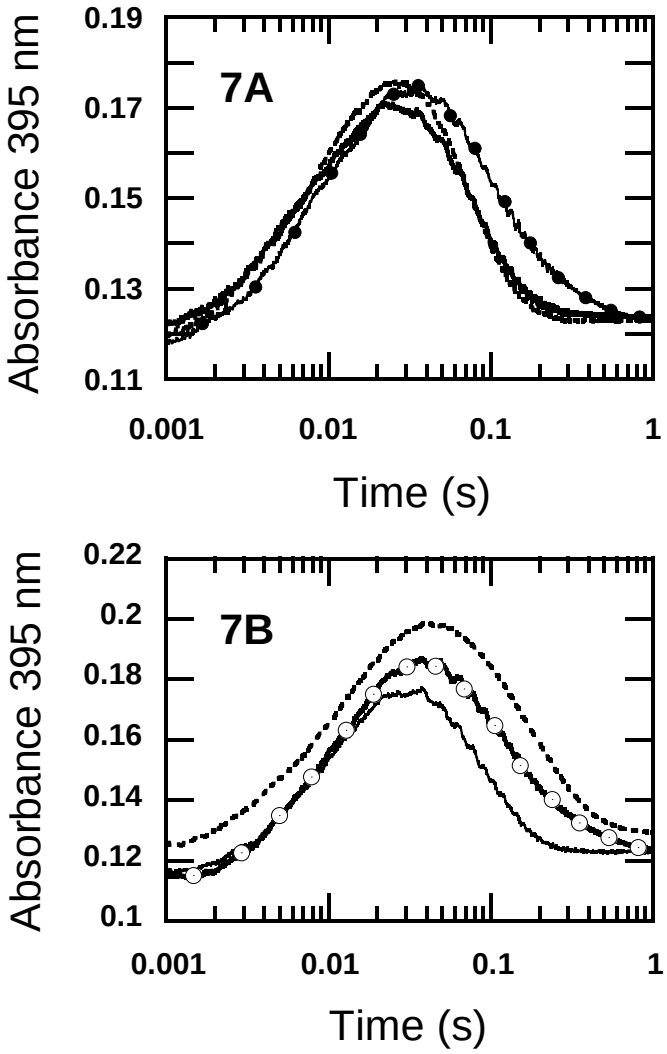

7A
Absorbance 395 nm
0.19
0.17
0.15
0.13
0.11
0.001
0.01
0.1
1
Time (s)
7B
Absorbance 395 nm
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.001
0.01
0.1
1
Time (s)

Figure 8

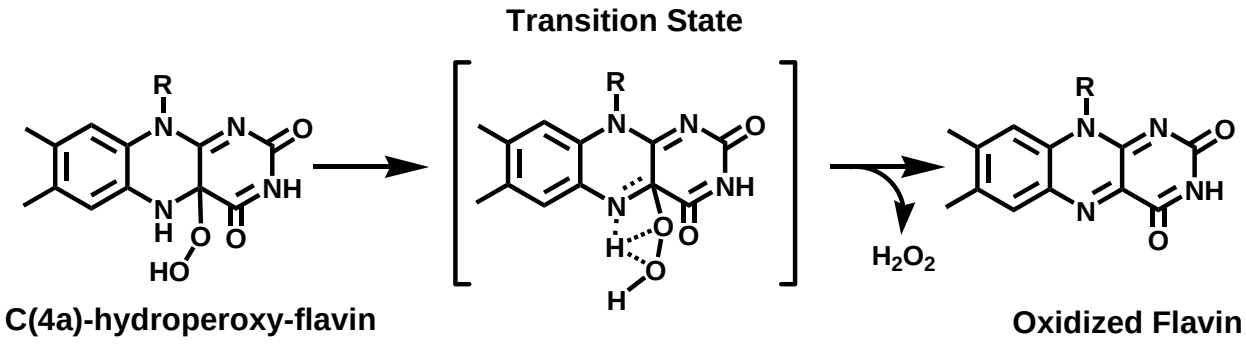

Transition State
C(4a)-hydroperoxy-flavin
H_2O_2
Oxidized Flavin

Figure 9

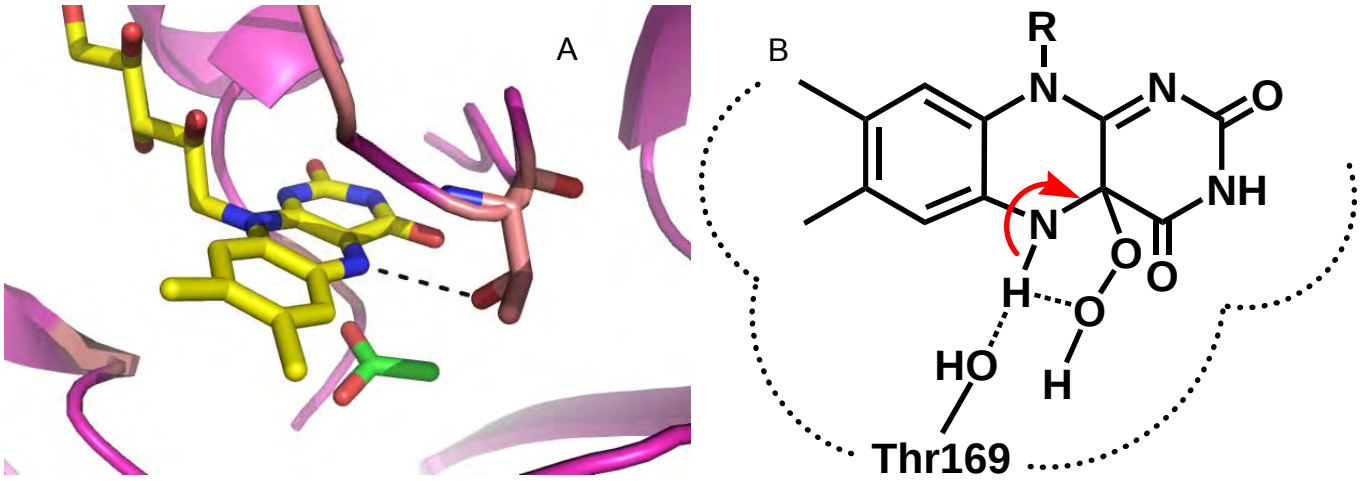


Table1. Effects of solvent isotope on the kinetics of P2O oxidative half-reaction. The reactions were performed in 100 mM sodium phosphate pH(D) 7.0 at 4 °C in stopped-flow spectrophotometer.

Parameters	H_2O buffer	D_2O buffer
k_1^{app} ($M^{-1}s^{-1}$)	$5.6 \pm 0.2 \times 10^4$	$5.9 \pm 0.3 \times 10^4$
k_1^{sim} ($M^{-1}s^{-1}$)	$6.5 \pm 0.3 \times 10^4$	$6.5 \pm 0.3 \times 10^4$
k_{-1}^{sim} (s^{-1})	2 ± 0.1	2 ± 0.1
k_2^{app} (s^{-1})	24 ± 2	6.9 ± 0.5
k_2^{sim} (s^{-1})	18 ± 1	6.4 ± 0.3

7. ภาคผนวก

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Sucharitakul J, Wongnate T, Chaiyen P (2011) Hydrogen peroxide elimination from C4a-hydroperoxyflavin in a flavoprotein oxidase occurs through a single proton transfer from flavin N5 to a peroxide leaving group. ***J. Biol. Chem. 286***, 16900-16909.

1.2 Sucharitakul J, Wongnate T, Montersino S, van Berkel WJ, Chaiyen P (2012) Reduction Kinetics of 3-Hydroxybenzoate 6-Hydroxylase from Rhodococcus jostii RHA1 (2012) ***Biochemistry*** [just accepted]

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงวิชาการ: ทำให้เกิดความรู้ใหม่ของกลไกปฏิกิริยา, วิธีวิจัยใช้เป็นต้นแบบในการทดลอง