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Kinetic Analysis of Nitrite Reduction Reactions by Nitrite Reductase Derived from Spinach in the Presence of One-Electron Reduced Riboflavin

Shusaku Ikeyama [1,*] and Hiroyasu Tabe [2]

[1] Institute of Advanced Research, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan
[2] Research Center for Artificial Photosynthesis, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan; htabe@ocarina.osaka-cu.ac.jp
* Correspondence: ikeyama@osaka-cu.ac.jp; Tel.: +81-(0)6-6605-3635

Abstract: The development of methods for converting nitrogen oxides in water into valuable resources such as ammonia and hydrazine has been given some attention. By utilizing the nitrite-reducing catalytic activity of nitrite reductase (NiR), nitrite in water can be converted into ammonium. However, there are few reports in the research that synthesized ammonium from nitrite using nitrite reductase. Therefore, we aimed to investigate the effect of temperature on the nitrite-reducing catalytic activity of NiR from spinach in the presence of one-electron reduced riboflavin by kinetic analysis to find the optimum temperature conditions. The results of this study showed that the reaction temperature does not need to be higher than 296.15 K in order to improve the efficiency of ammonium production from nitrite using NiR.

Keywords: nitrite reductase; nitrite-reducing catalytic activity; nitrite reduction; ammonium production; riboflavin

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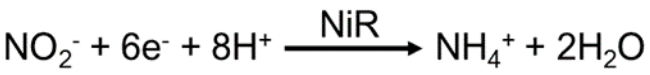
1. Introduction

The employment of the Haber–Bosch method (H-B method) solved the problem of reactive nitrogen supply (such as urea) and allowed human societies to benefit from improved food productivity. The H-B method will continue to be indispensable for nitrogen fixation. However, the utilization efficiency of fertilizers obtained from ammonia (NH_3) using the H-B method is low, with about 20% of reactive nitrogen being retained, while the remaining 80% leaks into the environment, causing environmental pollution [1–4]. In addition, to replace the amount of reactive nitrogen species (RNS) leaked into the environment, it is necessary to produce ammonia using the H-B method continuously. The H-B method leads to an increase in CO_2 emissions. Therefore, to continue with sustainable development, it is necessary to reuse the leaked RNS as much as possible and reduce CO_2 emissions. Nitrate and nitrite in aqueous solutions cause water pollution phenomena such as acidification [5–8]. Currently, RNS from solutions are converted into nitrogen using a biological denitrification method at sewage treatment plants and are then released into the atmosphere [9–11]. However, as much as possible, it is desirable to reuse reactive nitrogen that is produced by consuming large amounts of energy (without converting it back to nitrogen) in order to reduce the environmental load. Therefore, the development of a method to convert the reactive nitrogen present in water into ammonium (NH_4^+) or hydrazine for reuse has received considerable attention in recent years [12–14].

One method involving ammonium production from nitrate (NO_3^-) in water, which uses the catalytic activity of nitrate reductase (NR) and nitrite reductase (NiR), has been increasingly investigated [15–17]. By utilizing the catalytic activity of the two reductases, nitrate is converted into ammonium via nitrite (NO_2^-). Of the two enzymes, NiR catalyzes

the nitrite reduction reaction to ammonium, as shown in Scheme 1 [18,19], and nitrite can be converted into ammonium under ambient conditions in vitro. In the scheme, six electrons are provided by co-enzymes (electron donors), which have the role of expressing the nitrite-reducing catalytic activity of NiR. NiR recognizes ferredoxin or nicotinamide adenine dinucleotide (NADH) as a physiological electron donor for nitrite reduction in vivo [20,21]. In addition, one-electron reduced viologen, flavin, and thiazine derivatives are recognized as physiological electron donors for ferredoxin-dependent NiR in vitro [22–24]. Some viologen and flavin derivatives are quickly reduced and converted into radicals after exposure to light, chemical, or electrical energy. Therefore, by using these two derivatives as non-physiological electron donors—instead of physiological electron donors—it is possible to establish an ammonium production system from nitrite based on NiR and light energy [25,26]. As previously mentioned, NiR can convert nitrite to ammonium under ambient conditions in vitro. To proceed with research on the use of NiR in artificial systems, it is necessary to study the temperature dependence of the nitrite-reducing catalytic activity of NiR in vitro. Thus, the present study focused on estimating and calculating these two aspects using one-electron reduced riboflavin. The ferredoxin-dependent NiR derived from spinach, which recognizes one-electron reduced riboflavin as an electron donor, was used as a sample. Furthermore, riboflavin is inexpensive and one of the commercially available flavin derivatives. The Michaelis constant, which indicates the substrate affinity between spinach-derived NiR and nitrite ions, has been reported to be approximately 100 μM (5 ppm), indicating that the NiR exhibits sufficient nitrite-reducing activity in the presence of nitrite of about 10 ppm [27,28]. No other catalyst reduces nitrite to ammonia under conditions of such nitrite concentration. Therefore, it is worth considering the use of NiR derived from spinach in artificial systems for RNS recycling. More specifically, the findings obtained through this study can be used as an index for evaluating the catalytic activity of NiR, especially when it is immobilized on a carrier such as mesoporous silica [29].



Scheme 1. Nitrite (NO_2^-) reduction to ammonium (NH_4^+) via the nitrite-reducing catalytic activity of nitrite reductase (NiR).

2. Materials and Methods

2.1. Materials and Chemicals

All chemicals were obtained from chemical companies and used without further purification. Sodium dithionite ($Na_2S_2O_4$), sodium nitrite ($NaNO_2$), sodium hydrogen carbonate ($NaHCO_3$), riboflavin, dithiothreitol (DTT), benzylsulfonyl fluoride (PMSF), potassium hydroxide (KOH), acetone, and ammonium assay kits were purchased from FUJIFILM Wako Pure Chemical Corporation. 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid disodium salt (EDTA) were provided by Nacalai Tesque. The bicinchoninic acid (BCA) protein assay kit and polyvinylpyrolidone were obtained from TAKARA Bio Inc. (Otsu, shiga, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. Spinach plants were purchased from a local market. Distilled water was used as a solvent.

2.2. Methods

2.2.1. Preparation of Nitrite Reductase in a Spinach Plant Sample

The preparation of nitrite reductase in a spinach plant sample was carried out by following the previously reported method [23]. A solution consisting of 163 mg of ethylenediaminetetraacetic acid (EDTA), 70 μL of 2-mercaptomethanol, 15.4 mg of DTT, 7.7 mg of benzylsulfonylfluoride, and 1.6 g of polyvinylpyroridone in 50 mM of pH 7.4 HEPES-KOH buffer solution was prepared. A volume of 100 mL of this solution was used per 100 g of spinach leaves. All subsequent procedures were carried out in a low-temperature room at 4 °C. The leaves were crushed using a blending mixer and were filtered through a non-

woven fabric to obtain a solution containing NiR. The mixture was centrifuged at 19,000 rpm for 15 min, and the supernatant was collected. Ice-cold acetone (−20 °C) was added to the recovered solution, and its final concentration was adjusted to 35% (*v*/*v*). This solution was again centrifuged at 19,000 rpm for 15 min, and the supernatant was collected. Ice-cold acetone was further added to the supernatant to obtain a final concentration of 75% (*v*/*v*). A third centrifugation phase followed, at 19,000 rpm for 15 min. After the supernatant was removed, the precipitate was collected, dried at room temperature for 5 min, and was then suspended in 50 mM of HEPES-KOH buffer solution at pH 7.4. The suspension was centrifuged at 19,000 rpm for 5 min, and finally, the supernatant was collected and ultrafiltered using a 50 kDa Amicon Ultra centrifugal filter unit. The molecular weight of the nitrite reductase derived from spinach was reported to be 61 kDa [30]. The supernatant containing over 50 kDa protein was collected. Protein concentration in the collected supernatant was adjusted by adding 50 mM of pH 7.4 HEPES-KOH buffer solution as a solvent. The protein concentration in the liquid was estimated using the bicinchoninic acid (BCA) method.

2.2.2. Activity Test of Nitrite Reductase Derived from Spinach with One-Electron Reduced Riboflavin as Electron Donor

Ammonium was produced from nitrite to estimate the activity of the spinach-derived NiR. The sample solution tested consisted of 4.7 mg/mL NiR-containing protein in a mixture, 64 μM of riboflavin, 2 mM of $NaNO_2$, and 30 mM of $Na_2S_2O_4$ in 50 mM of pH 7.4 HEPES-KOH buffer. Riboflavin was used as an electron donor and $Na_2S_2O_4$ as a reducer for riboflavin in the activity test. In addition, 74 mM of $NaHCO_3$ was added to prevent acidification of the sample solution caused by $Na_2S_2O_4$, and to keep its pH at 7.4; its volume was adjusted to 0.4 mL. The sample solution was placed in a 1.5 mL microtube and was reacted using an Eppendorf shaker at a temperature range between 288.15 and 303.15 K for 1 min. The ammonium production was initiated by adding the $Na_2S_2O_4$ solution, and the amount of ammonium produced was estimated using the indophenol blue method. The absorption spectrum of the solutions used for ammonium detection was measured using a quartz cell and a spectrophotometer (UV-1800, SHIMADZU, Kyoto, Japan).

3. Results and Discussions

3.1. Temperature Dependence of Nitrite-Reducing Catalytic Activity

The temperature dependence of the nitrite-reducing activity of the spinach-derived NiR was estimated. The activity test was performed three times under different temperature conditions. Figure 1 shows the relationship between the reaction temperature and the ammonium production rate (Detailed values are listed in Table A1). Ammonium produced between 288.15 and 296.15 K increased with the increasing reaction temperature. In particular, the highest ammonium production was obtained at 296.15 K. In contrast, the ammonium production rate decreased as temperature increased above 296.15 K. As ammonium production from nitrite is an exothermic reaction, the results of the temperature dependence test of the nitrite-reducing catalytic activity of the NiR derived from spinach and one-electron reduced riboflavin that was conducted in this study are valid [31]. Despite this fact, the rate of ammonium production increased in proportion to the temperature rise between 288.15 and 296.15 K. One of the reasons for this increased production is the substrate diffusion rate between NiR and one-electron reduced riboflavin. Under all conditions, the nitrite concentration before the reaction was 2000 μM, and there was a sufficient amount of NiR to express nitrite-reducing catalytic activity. In contrast, the riboflavin concentration was 64 μM in the reaction solution because riboflavin is difficult to dissolve in water. In reducing nitrite to ammonium with NiR, six electrons were required to reduce one molecule of nitrite. In other words, six molecules of one-electron reduced riboflavin were necessary for the reaction to occur. Therefore, under reaction temperatures below 296.15 K, the stage of the electron transfer to NiR via one-electron reduced riboflavin was considered to be the rate-determining step and the cause of the decreased ammonium production rate. Clearly, it is necessary to control the temperature and improve the collision

frequency between the electron carrier and the NiR to improve efficiency of the reaction. As in one method, flavin derivatives that are more soluble in water than riboflavin can increase the abundance of electron carriers in the reaction solution. This will contribute to the increase in ammonium production from nitrite using NiR.

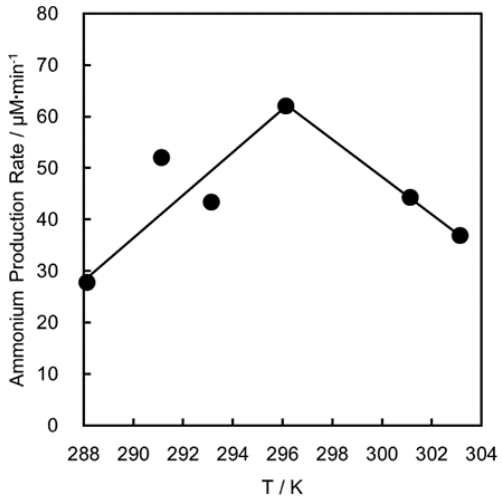


Figure 1. Temperature dependence of the nitrite-reducing activity of NiR derived from spinach.

3.2. Calculation of the Activation Energy in Ammonium Production

The activation energy (E_a) was calculated, based on the results of the activity test, to be between 288.15 and 296.15 K. The rate constant value (k) was calculated based on the following formula:

$$k = \frac{\left[NH_4{}^+\right]\left[H_2O\right]^2}{\left[NO_2{}^-\right]\left[H^+\right]^8}$$

As the pH of the reaction solution was stable, the proton concentration ($[H^+]$) in the formula was set to 1.

Figure 2 depicts the Arrhenius plots showing the relationship between the logarithmic values of the rate constant ($\ln k$)—calculated based on the above equation—and the reciprocal values of the reaction temperature ($10^3 \times 1/T$) (The calculated values are listed in Table A2). The value for the straight line obtained from these plots was 19.985. The activation energy calculated from the slope value was 166.1 kJ mol^{-1}. The calculated E_a value is helpful in investigating the effect of each element involved in the reaction on the nitrite-reducing catalytic activity of NiR, particularly when the reaction conditions are changed in ammonia production using NiR. More specifically, the E_a value is expected to serve as an index for comparing the catalytic activity of NiR when NiR is immobilized using various inorganic materials as a carrier.

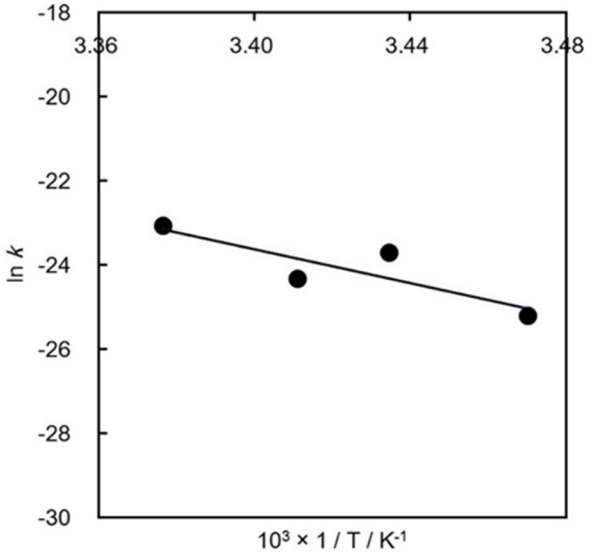


Figure 2. Arrhenius plots of experimental and calculated rate constants for the reduction of nitrite to ammonium with NiR derived from spinach.

4. Conclusions

In the present study, the activation energy (E_a) of ammonium production from nitrite using spinach-derived NiR and one-electron reduced riboflavin was calculated for the first time (E_a = 166.1 kJ mol^{-1}). NiR shows high activity at 296.15 K, suggesting that additional thermal energy inputs are not needed to improve reaction efficiency. Through NiR activity, it is possible to obtain ammonium from nitrite with a low environmental load. Based on the indicators obtained through this research, we will investigate the efficient use of the nitrite-reducing catalytic activity of NiR in vitro by immobilizing NiR using an inorganic material such as mesoporous silica as a carrier.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Values obtained from the activity tests with nitrite reductase derived from spinach and one-electron reduced riboflavin.

***T*: Reaction Temperature K**	***v*: Ammonium Production Rate μM·min^{-1}**
288.15	27.7
291.15	52.0
293.15	43.4
296.15	62.0
301.15	44.2
303.15	36.8

The sample solution of the activity test consisted of 4.7 mg/mL of spinach-derived NiR, 64 µM of riboflavin, 8 mM of sodium nitrite ($NaNO_2$), 30 mM of sodium dithionite ($Na_2S_2O_4$), and 74 mM of sodium hydrogen carbonate ($NaHCO_3$) in 50 mM of HEPES-KOH buffer at pH 7.4. The volume of the sample solution was 0.4 mL. The ammonium production reaction time was 1 min. Each ammonium production rate (v) reported is the average production rate values obtained from three activity tests.

Table A2. Values used to generate the Arrhenius plots.

$1/T$ K^{-1}	k: Rate Constant $10^{-12} \times M^2$	ln k
3.47	10.8	−25.2
3.43	72.2	−23.7
3.41	41.5	−24.3
3.38	123.0	−23.1

References

1. Stevens, C.J. Nitrogen in the environment. *Science* **2019**, *363*, 578–580. [CrossRef] [PubMed]
2. Zhang, X.; Wu, Y.; Gu, B. Urban rivers as hotspots of regional nitrogen pollution. *Environ. Pollut.* **2015**, *205*, 139–144. [CrossRef] [PubMed]
3. Prasad, R. Fertilizer nitrogen, food security, health and the environment. *World* **2013**, *16*, 14–16.
4. Choudhury, A.T.M.A.; Kennedy, I.R. Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. *Commun. Soil. Sci. Plant Anal.* **2005**, *36*, 1625–1639. [CrossRef]
5. Schullehner, J.; Hansen, B.; Thygesen, M.; Pedersen, C.B.; Sigsgaard, T. Nitrate in drinking water and colorectal cancer risk: A nationwide population-based cohort study. *Int. J. Cancer* **2018**, *143*, 73–79. [CrossRef] [PubMed]
6. Pisciotta, A.; Cusimano, G.; Favara, R. Groundwater nitrate risk assessment using intrinsic vulnerability methods: A comparative study of environmental impact by intensive farming in the Mediterranean region of Sicily, Italy. *J. Geochem. Explor.* **2015**, *156*, 89–100. [CrossRef]
7. Archer, M.C. Hazards of Nitrote, Nitrite, ond N-Nitroso Compounds in Human Nutrition. *Nutr. Toxicol. V1* **2012**, *1*, 327.
8. Kroupova, H.; Machova, J.; Svobodova, Z. Nitrite influence on fish: A review. *Vet. Med.-Praha-* **2005**, *50*, 461–471. [CrossRef]
9. Rezvani, F.; Sarrafzadeh, M.H.; Ebrahimi, S.; Oh, H.M. Nitrate removal from drinking water with a focus on biological methods: A review. *Environ. Sci. Pollut. Res.* **2019**, *26*, 1124–1141. [CrossRef]
10. Gayle, B.P.; Boardman, G.D.; Sherrard, J.H.; Benoit, R.E. Biological denitrification of water. *J. Environ. Chem. Eng.* **1989**, *115*, 930–943. [CrossRef]
11. Wuhrmann, K. Nitrogen removal in sewage treatment processes: Int. Ver. Theor. *Angew. Limnol.* **1964**, *15*, 580–596.
12. Liang, J.; Deng, B.; Liu, Q.; Wen, G.; Liu, Q.; Li, T.; Sun, X. High-efficiency electrochemical nitrite reduction to ammonium using a Cu3P nanowire array under ambient conditions. *Green Chem.* **2021**, *23*, 5487–5493. [CrossRef]
13. Clark, C.A.; Reddy, C.P.; Xu, H.; Heck, K.N.; Luo, G.; Senftle, T.P.; Wong, M.S. Mechanistic insights into pH-controlled nitrite reduction to ammonia and hydrazine over rhodium. *ACS Catal.* **2019**, *10*, 494–509. [CrossRef]
14. Guo, Y.; Stroka, J.R.; Kandemir, B.; Dickerson, C.E.; Bren, K.L. Cobalt metallopeptide electrocatalyst for the selective reduction of nitrite to ammonium. *J. Am. Chem. Soc.* **2018**, *140*, 16888–16892. [CrossRef]
15. Sachdeva, V.; Hooda, V. Immobilization of nitrate reductase onto epoxy affixed silver nanoparticles for determination of soil nitrates. *Int. J. Biol. Macromol.* **2015**, *79*, 240–247. [CrossRef]
16. Sachdeva, V.; Hooda, V. Effect of changing the nanoscale environment on activity and stability of nitrate reductase. *Enzyme Microb. Technol.* **2016**, *89*, 52–62. [CrossRef]
17. Andoralov, V.; Shleev, S.; Dergousova, N.; Kulikova, O.; Popov, V.; Tikhonova, T. Octaheme nitrite reductase: The mechanism of intramolecular electron transfer and kinetics of nitrite bioelectroreduction. *Bioelectrochemistry* **2021**, *138*, 107699. [CrossRef]
18. Einsle, O.; Messerschmidt, A.; Huber, R.; Kroneck, P.M.; Neese, F. Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome c nitrite reductase. *J. Am. Chem. Soc.* **2002**, *124*, 11737–11745. [CrossRef]
19. Einsle, O.; Messerschmidt, A.; Stach, P.; Bourenkov, G.P.; Bartunik, H.D.; Huber, R.; Kroneck, P.M. Structure of cytochrome c nitrite reductase. *Nature* **1999**, *400*, 476–480. [CrossRef]
20. Wang, X.; Tamiev, D.; Alagurajan, J.; DiSpirito, A.A.; Phillips, G.J.; Hargrove, M.S. The role of the NADH-dependent nitrite reductase, Nir, from *Escherichia coli* in fermentative ammonification. *Arch. Microbiol.* **2019**, *201*, 519–530. [CrossRef]
21. Kuznetsova, S.; Knaff, D.B.; Hirasawa, M.; Lagoutte, B.; Sétif, P. Mechanism of spinach chloroplast ferredoxin-dependent nitrite reductase: Spectroscopic evidence for intermediate states. *Biochemistry* **2004**, *43*, 510–517. [CrossRef] [PubMed]
22. Xuejiang, W.; Dzyadevych, S.V.; Chovelon, J.M.; Renault, N.J.; Ling, C.; Siqing, X.; Jianfu, Z. Conductometric nitrate biosensor based on methyl viologen/Nafion®/nitrate reductase interdigitated electrodes. *Talanta* **2006**, *69*, 450–455. [CrossRef]
23. Ida, S.; Morita, Y. Purification and general properties of spinach leaf nitrite reductase. *Plant Cell Physiol.* **1973**, *14*, 661–671.

24. Bourne, W.F.; Miflin, B.J. Studies on nitrite reductase in barley. *Planta* **1973**, *111*, 47–56. [CrossRef] [PubMed]
25. Willner, I.; Willner, B. Artificial photosynthetic transformations through biocatalysis and biomimetic systems. *Adv. Photochem.* **1995**, *20*, 217–290.
26. Willner, I.; Lapidot, N.; Riklin, A. Photoinduced enzyme-catalyzed reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to ammonia (NH_3). *J. Am. Chem. Soc.* **1989**, *111*, 1883–1884. [CrossRef]
27. Ramirez, J.M.; Del Campo, F.F.; Paneque, A.; Losada, M. Ferredoxin-nitrite reductase from spinach. *Biochim. Biophys. Acta.* **1966**, *118*, 58–71. [CrossRef]
28. Hirasawa-Soga, M.; Tamura, G. Some properties of ferredoxin-nitrite reductase from Spinacia oleracea. *Agric. Biol. Chem.* **1981**, *45*, 1615–1620. [CrossRef]
29. Tabe, H.; Oshima, H.; Ikeyama, S.; Amao, Y.; Yamada, Y. Enhanced catalytic stability of acid phosphatase immobilized in the mesospaces of a SiO_2-nanoparticles assembly for catalytic hydrolysis of organophosphates. *Mol. Catal.* **2021**, *510*, 111669. [CrossRef]
30. Vega, J.M.; Kamin, H. Spinach nitrite reductase. Purification and properties of a siroheme-containing iron-sulfur enzyme. *J. Biol. Chem.* **1977**, *252*, 896–909. [CrossRef]
31. Bykov, D.; Neese, F. Six-electron reduction of nitrite to ammonia by cytochrome c nitrite reductase: Insights from density functional theory studies. *Inorg. Chem.* **2015**, *54*, 9303–9316. [CrossRef] [PubMed]