[Regular Paper]

Photochemical and Enzymatic Synthesis of Malic Acid from Pyruvic Acid and HCO$_3^-$ with Combination System of Zinc Chlorin-e$_6$ and Malic Enzyme in Aqueous Medium

Yutaka AMAO* and Mitsue ISHIKAWA

Dept. of Applied Chemistry, Oita University, Dannohara 700, Oita 870-1192, JAPAN

(Received January 9, 2007)

Photochemical and enzymatic synthesis of malic acid from pyruvic acid and HCO$_3^-$ was studied with malic enzyme (ME) and NADP$^+$ photoreduction with ferredoxin-NADP$^+$-reductase (FNR) by visible light photosensitization of the chlorophyll derivative, zinc chlorin-e$_6$ (ZnChl-e$_6$), in the presence of NADH as an electron-donating reagent and methyliologen (MV$^{2+}$) as an electron-relay reagent. NADPH was produced by irradiation of the reactant solution containing NADH, ZnChl-e$_6$, MV$^{2+}$, NADP$^+$ and FNR in Bis-Tris buffer (pH 8.0). After 180-min irradiation, 8.3 mmol·dm$^{-3}$ NADPH was produced under the optimum conditions with 10 mmol·dm$^{-3}$ of NADP$^+$. The reduction ratio of NADP$^+$ to NADPH was about 83%. Malic acid was produced by irradiation with visible light (>390 nm) of the reactant solution containing NADH, ZnChl-e$_6$, methyliologen (MV$^{2+}$), pyruvic acid, NaHCO$_3$, NADP$^+$, ferredoxin NADP$^+$ reductase (FNR) and ME. Malic acid production was 2.0 mmol·dm$^{-3}$ after 3-h irradiation under the optimum conditions with NADH, ZnChl-e$_6$, MV$^{2+}$, FNR, pyruvic acid, NaHCO$_3$, NADP$^+$ and ME. The ratio of HCO$_3^-$ and pyruvic acid to malic acid was about 20% under the optimum conditions.

Keywords
Malic acid synthesis, Zinc chlorin-e$_6$, Malic enzyme, Enzymatic synthesis, Water medium

1. Introduction

CO$_2$ fixation is a potential technology for the realization of photocatalytic CO$_2$ reduction and synthesis of organic compounds from CO$_2$ as a starting material. Many studies of CO$_2$ fixation or utilization have investigated ultra-visible light induced photocatalysis on semiconductors such as titanium dioxide$^{[1]}$, silicon carbide$^{[1]}$ and strontium titanate$^{[2]}$ in heterogeneous aqueous media. Biological CO$_2$ fixation or utilization systems have also received much attention, because organic compounds can be synthesized in homogenous aqueous media by using water soluble enzymes with CO$_2$ fixation function. For example, CO$_2$ or HCO$_3^-$ can be reduced to formic acid with formate dehydrogenase (FDH) and NADH. Therefore, a CO$_2$ fixation or utilization system that combines the photoreduction of NAD$^+$ with photosensitizer and ferredoxin-NADP$^+$-reductase (FNR), and HCO$_3^-$ reduction with FDH can be established. We previously reported visible-light induced enzymatic formic acid synthesis from CO$_2$ or HCO$_3^-$ with FDH using the photosensitization of water-soluble zinc porphyrin and chlorophyll$^{[17,19]}$. CO$_2$ fixation based on malic acid synthesis combined with the photoreduction of NAD$^+$ by the photosensitization of ruthenium(II) polypyridyl complex or chemically modified chlorophyll and ferredoxin-NADP$^+$-reductase (FNR), and malic acid synthesis from pyruvate and HCO$_3^-$ with malic enzyme (ME) have also been reported$^{[15,16]}$. In this system, the carbon chain is extended from C3 (pyruvate) to C4 (malic acid) by photochemical and enzymatic methods in the aqueous medium. To further improve the reaction efficiency using ruthenium(II) polypyridyl complexes, near-infrared absorption must be improved, which is weak because of the small extinction coefficient of such ruthenium(II) complexes above 600 nm.

Chlorophylls, which act as the effective photosensitizer in the photosynthesis reaction$^{[17]}$, and derivatives such as chlorin-e$_6$ have absorption bands in the visible region. Thus, chlorophylls have high potential as visible light photosensitizers. Chlorophyll molecules are insoluble in aqueous media, but chlorin-e$_6$ (Chl-e$_6$) is formed by the hydrolysis of chlorophyll-a and contains three carboxylate groups in its molecule. Water-soluble zinc porphyrins also have absorption bands in the visible light region (380-600 nm), and are widely used as effective photosensitizers$^{[18]}$. Zinc tetraphenylporphyrin tetraysulfonate (ZnTPPS) and zinc tetrakis(4-methylpyridyl)porphyrin (ZnTMPyP) are

* To whom correspondence should be addressed.
* E-mail: amao@cc.oita-u.ac.jp
especially useful as photosensitizers\(^{13-15}\). We previously reported visible-light induced enzymatic formic acid synthesis from CO\(_2\) or HCO\(_3^-\) with FDH using the photosensitization of ZnTMPyP\(^{31-36}\). We also reported visible-light induced enzymatic methanol synthesis from HCO\(_3^-\) or formaldehyde with dehydrogenases using the photosensitization of ZnTPPS\(^{16-18}\). However, the molar absorption coefficient (\(\varepsilon\)) of zinc porphyrins in the visible light region (500-600 nm) is lower than that in the near ultra-visible light region (380-400 nm). In contrast, the zinc complex of Chl-e\(_6\) (ZnChl-e\(_6\)) has an absorption maximum at 638 nm (\(\varepsilon=4.0\times10^4\) mol \(^{-1}\) dm\(^3\) cm\(^{-1}\)). We reported the development of photochemical and enzymatic malic acid synthesis from HCO\(_3^-\) and pyruvic acid with ME, via the photoreduction of NADP\(^+\) using ZnChl-e\(_6\), photosensitization\(^{19}\). However, the detailed reaction of the photochemical and enzymatic malic acid synthesis has not been clarified yet.

The present study investigated visible-light induced malic acid synthesis from pyruvic acid and HCO\(_3^-\) with ME using the photosensitization of zinc chlorin-e\(_6\) (ZnChl-e\(_6\)) as shown in Scheme 1.

2. Experimental

2.1. Materials

ME from chicken liver and FNR from Spinacia oleracea were obtained from Sigma Co., Ltd. Methyl viologen dichloride (MV\(^2+\)) was supplied by Tokyo Chem. Ind. Co., Ltd. Chl-e\(_6\) was purchased from Wako Pure Chemical Industries, Ltd. NADP\(^+\) and NADH were purchased from Oriental Yeast Co., Ltd. The other chemicals were analytical grade or the highest grade available. One unit of ME activity was defined as the amount of enzyme required to produce 1.0 mmol malic acid to HCO\(_3^-\) and pyruvic acid per min in the presence of NADP\(^+\).

2.2. Synthesis of Zinc Chlorin-e\(_6\) (ZnChl-e\(_6\))

ZnChl-e\(_6\) was synthesized by refluxing Chl-e\(_6\) with about 10 times the molar equivalent of zinc acetate in 100 ml methanol at 60°C for 2 h according to the previously reported method\(^{20}\). The synthesis of ZnChl-e\(_6\) was monitored by UV-vis absorption spectrum using a Shimadzu MultiSpec 1500 spectrophotometer. During the reaction, the characteristic absorption bands of ZnChl-e\(_6\) at 418 and 638 nm increased and the absorbance at 400, 514 and 660 nm of Chl-e\(_6\) decreased gradually. After the mixture was cooled to room temperature, the solvent was removed under vacuum and then the reaction mixture was washed with water to remove the unreacted zinc acetate dihydrate. Finally, ZnChl-e\(_6\) was precipitated in water. ZnChl-e\(_6\) was collected by filtration and washed with water. The product was recrystallized from water-methanol (5:1) solution.

2.3. Photoreduction of MV\(^2+\) by Photosensitization of ZnChl-e\(_6\)

A solution containing ZnChl-e\(_6\), MV\(^2+\) and electron donating reagent in 3.0 ml of Bis-Tris buffer (pH 8.0) was deoxygenated by freeze-pump-thaw cycles repeated 6 times. The sample solution was irradiated with a 200 W tungsten lamp (Philips) at a distance of 3.0 cm. The light intensity was 80 mW cm\(^{-2}\) measured with a laser power meter model AN/2 Cophir Optronics, Inc. To prevent degradation of ZnChl-e\(_6\) by UV light, wavelengths of less than 390 nm were blocked with a Toshiba L-39 cut-off filter (process 1 in Scheme 1). Reduced MV\(^2+\) (MV\(^{2+}\)) concentration was determined by the absorbance at 605 nm using the molar coefficient 1.3 \times 10^9 mol \(^{-1}\) dm\(^{-1}\) cm\(^{-1}\). To identify the optimum electron-donating reagent, ascorbic acid, triethanolamine (TEOA) and NADH were used. The MV\(^2+\) concentrations in the solution were varied between 0.015 and 0.24 nmol dm\(^{-3}\).

2.4. Photoreduction of NADP\(^+\) via the Reduction of MV\(^2+\) by Photosensitization of ZnChl-e\(_6\)

A solution containing NADH, ZnChl-e\(_6\), MV\(^2+\) and FNR in Bis-Tris buffer (pH 8.0) was deoxygenated by freeze-pump-thaw cycles repeated 6 times and then flushed with argon gas for 5 min. NADP\(^+\) solution was added to the sample solution and then flushed with argon gas for 5 min. The sample solution was irradiated with a 200 W tungsten lamp and wavelengths of less than 390 nm were blocked with a cut-off filter (Toshiba L-39) at 30°C (processes 1 and 2 in Scheme 1). The light intensity was 80 mW cm\(^{-2}\). NADPH produced was detected by HPLC with an electrical conductivity detector (Shimadzu CDD-10A vp) (column temperature: 40°C, column: polystyrene sulfonate column Shimadzu).

SCR-H, eluant: p-toluene sulfonic acid and flow rate: 0.8 mL/min). The NADP⁺ concentrations in the solution were 0, 0.1 and 10 mmol·dm⁻³.

2.5. Photochemical and Enzymatic Synthesis of Malic Acid from HCO₃⁻ and Pyruvic Acid

A solution containing NADH, ZnChl-ε₈, MV²⁺, FNR, pyruvic acid and NaHCO₃ in Bis-Tris buffer (pH 8.0) was deaerated by freeze-pump-thaw cycles repeated 6 times and then flushed with argon gas for 5 min. NADP⁺ solution and ME were added to the sample solution and then flushed with argon gas for 5 min. The sample solution was irradiated with a 200 W tungsten lamp and wavelengths of less than 390 nm were blocked with a cut-off filter at 30°C (Scheme 1). The light intensity was 80 mW·cm⁻². Malic acid produced was detected by HPLC with an electrical conductivity detector (column temperature: 40°C, column: polystyrene sulfonate column Shimadzu SCR-H, eluant: p-toluene sulfonic acid and flow rate: 0.8 mL/min). The NADP⁺ concentrations in the solution were varied between 0 and 10 mmol·dm⁻³.

3. Results and Discussion

3.1. Reduction of MV²⁺ with Visible Light Sensitization of ZnChl-ε₈

To investigate the optimum electron-donating reagent in the malic acid synthesis system, the MV²⁺ reduction system was studied with visible light sensitization of ZnChl-ε₈ in the presence of ascorbic acid, TEOA or NADH as an electron donor. The time dependence of the MV⁺ concentration in the system containing ZnChl-ε₈, MV²⁺ and TEOA under visible light irradiation is shown in Fig. 1. The absorbance at 605 nm attributed to the absorption band of MV⁺ increased with irradiation time. After 60 min irradiation, the concentrations of MV⁺ produced with NADH, TEOA and ascorbic acid were 56, 1.4 and 2.3 μmol·dm⁻³, respectively. In all cases, each data point represents the mean of at least five separate experiments and the error was less than 2%. By using NADH as an electron donor, the MV²⁺ reduction rate proceeded effectively. Thus, NADH was the optimum electron-donating reagent for the MV²⁺ reduction system with visible light sensitization of ZnChl-ε₈. The effect of MV²⁺ concentration on the time dependence of MV⁺ formation in the system containing ZnChl-ε₈, MV²⁺ and NADH with visible light irradiation is shown in Fig. 2. The rate of MV⁺ formation increased with the concentration of MV²⁺. At a MV²⁺ concentration of 0.24 mmol·dm⁻³, the conversion yield of MV²⁺ to MV⁺ was 23.5%. The rate of MV⁺ formation was important for malic acid synthesis using the system shown in Scheme 1. Therefore, the optimum MV²⁺ concentration was 0.24 mmol·dm⁻³ for malic acid synthesis.

![Fig. 1 Time Dependence of MV²⁺ Reduction under Steady State Irradiation with Visible Light Using a 200 W Tungsten Lamp at a Distance of 3.0 cm](image1)

![Fig. 2 Time Dependence of MV²⁺ Reduction under Steady State Irradiation with Visible Light Using a 200 W Tungsten Lamp at a Distance of 3.0 cm](image2)

3.2. Photoreduction of NADP⁺ via the Reduction of MV²⁺ by Photosensitization of ZnChl-ε₈

The time dependence of the NADPH concentration during irradiation of the solution containing NADH, ZnChl-ε₈, MV²⁺, NADP⁺ and FNR in 50 mmol·dm⁻³ of
Bis-Tris buffer (pH 8.0) is shown in Fig. 3. In all cases, each data point represents the mean of at least five separate experiments and the error was less than 2%. The rate of NADPH formation increased with the concentration of NADP⁺. After 180 min irradiation, 0.35 and 8.3 mmol·dm⁻³ NADPH were produced under NADP⁺ concentrations of 0.1 and 10 mmol·dm⁻³, respectively. The reduction ratio of NADP⁺ to NADPH was about 3.5 and 83% under NADP⁺ concentrations of 0.1 and 10 mmol·dm⁻³, respectively. These results indicate that effective NADP⁺ photoreduction with FNR was developed via MV²⁺ reduction with photosensitization of ZnChl-e₈. However, as irradiation will cause the decomposition of NADP⁺ or NADPH, the reduction ratio of NADP⁺ to NADPH cannot reach 100%. Moreover, no NADP⁺ photoreduction was observed without FNR, MV²⁺ or irradiation.

3.3. Photochemical and Enzymatic Synthesis of Malic Acid from HCO₃⁻ and Pyruvic Acid

After development of the NADP⁺ photoreduction system with photosensitization of ZnChl-e₈, photochemical malic acid synthesis from HCO₃⁻ and pyruvic acid was attempted using the above conditions with ME. Irradiation of a solution containing NADH, ZnChl-e₈, MV²⁺, FNR, pyruvic acid, NaHCO₃, NADP⁺ and ME in Bis-Tris buffer (pH 8.0) resulted in the concentrations of malic acid and pyruvic acid as shown in Figs. 4(a) and 4(b), respectively. In all cases, pyruvic acid decreased with increased malic acid production under continuous irradiation. Therefore, photoinduced conversion of HCO₃⁻ and pyruvic acid to malic acid was achieved. The rate of malic acid formation decreased with the concentration of NADP⁺. The malic
acid concentration was 2.0 mmol·dm⁻³ after 3 h irradiation under NADP⁺ concentration of 0.01 mmol·dm⁻³. The ratio of HCO₃⁻ and pyruvic acid to malic acid was about 20%. The photochemical and enzymatic synthesis of malic acid from pyruvic acid and HCO₃⁻ with a system containing ascorbate as an electron donating reagent, chemically modified chlorophyll as a photosensitizer, NAD⁺, FNR, NADP⁺ and ME was reported previously. In this study, malic acid synthesis with ME and direct photoreduction of NADP⁺ by the photosensitization of chemical modified chlorophyll in the absence of MV²⁺ was attempted and the ratio of pyruvic acid to malic acid was about 11.1%. Thus, effective photochemical synthesis of malic acid from HCO₃⁻ and pyruvic acid was achieved with ME and NADPH produced by the photosensitization of ZnChl-ε₀ in the presence of MV²⁺. No synthesis of malic acid was observed without FNR (open circle), MV²⁺ (open triangle) or irradiation (×). As the NADH substrate for FNR, malic acid will be produced in the absence of ZnChl-ε₀. However, malic acid reached 0.03 mmol·dm⁻³ in the absence of ZnChl-ε₀ under irradiation ([NADP⁺] = 0.1 mmol·dm⁻³) as shown in Fig. 4 (open triangle). These results show that malic acid synthesis proceeds by coupling the NADP⁺ reduction using the photosensitization of ZnChl-ε₀ and enzymatic synthesis of malic acid with ME via the enzymatic function of FNR in aqueous medium.

The effect of light wavelength on the rates of malic acid synthesis with ZnChl-ε₀ was investigated. Irradiation with 200 W tungsten lamp through a Sigma sharp-cut filter SCF-50S-60R (transmitted above the wavelength of 600 nm) of a solution containing NADH, ZnChl-ε₀, MV²⁺, FNR, pyruvic acid, NaHCO₃, NADP⁺ and ME in Bis-Tris buffer (pH 8.0) resulted in the concentrations of malic acid and pyruvic acid as shown in Fig. 4 (closed square) ([NADP⁺] = 0.1 mmol·dm⁻³). After 180 min irradiation, 1.8 mmol·dm⁻³ malid acid was produced. The amount of malic acid production only decreased by 20% using irradiation with wavelengths above 600 nm. This result shows that an effective visible and near IR light induced malic acid synthesis system was established using the photosensitization of ZnChl-ε₀.

Acknowledgment

This work was partially supported by the "Grant for Research" of The Japan Petroleum Institute.

References

要　旨

水を反応媒体としたリンゴ酸酵素-亜鉛クロリン系によるビルビン酸
および炭酸水素イオンを原料とした光化学的リンゴ酸合成

天尾　豊，石川　満枝

大分大学工学部応用化学科，870-1192 大分市旦野原700

リンゴ酸酵素による炭酸水素イオンとビルビン酸からのリン
ゴ酸への変換反応と水溶性亜鉛クロリン系（ZnChl-ε6）の光増
感作用による NADP⁺の光還元反応とを組み合わせた光化学的
リンゴ酸合成系を構築した。具体的には電子供与体である
NADH，ZnChl-ε6，メチルビオローゲン（MV⁺），フェレドキ
シン-NADP⁺還元酵素（FNR），NADP⁺およびリンゴ酸酵素（ME）
からなる系である。この反応系に炭酸水素ナトリウムとビルビ
ン酸を添加し，200 W タングステンランプを用い可視光を照射
すると光照射時間とともに定常的にリンゴ酸が生成した。また,
リンゴ酸の生成とともにビルビン酸の減少が観測された。リン
ゴ酸合成系の反応条件を検討した結果，反応条件 NADH
（30 mmol·dm⁻³），ZnChl-ε6（50 µmol·dm⁻³），MV⁺
（0.24 mmol·dm⁻³），FNR（4.0 units），ビルビン酸（10 mmol·dm⁻³），
NaHCO₃（10 mmol·dm⁻³），NADP⁺（0.01 mmol·dm⁻³）および
ME（4.5 units）のときに，光照射3時間でリンゴ酸生成量は
2.0 mmol·dm⁻³であり，ビルビン酸からリンゴ酸への変換率は
20％であった。

-----------------------------------------------