Resazurin as an Electron Acceptor in Glucose Oxidase-Catalyzed Oxidation of Glucose

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The behavior of resazurin (1) as an electron acceptor in glucose oxidase (GOD)-catalyzed oxidation of glucose under anaerobic conditions is described. When a mixture of 1, glucose, and GOD in phosphate buffer (pH 7.4, 0.1 M) was incubated at 25 °C, the resulting solution turned purple to fluorescent pink due to the deoxygenated product, resorufin (2). On incubation of 1 with GOD alone or with H_2O_2 under essentially the same conditions, no color change was seen, indicating that generation of 2 in the enzymatic reaction is brought about through reduction of 1 by the reduced form (GOD_{red}) of GOD, which was also supported by the voltammetric behavior of 1. However, it was found that the enzymatic transformation of 1 to 2 is of no practical use as an indicator reaction for glucose determination using only GOD due to a slow reaction of 1 with GOD_{red}. Based on a ping-pong type mechanism with a steady-state approximation, K_M and k_{cat} for 1 as an electron acceptor from GOD_{red} were estimated to be 15 ± 1.3 μM and $(5.0\pm0.5)\times10^{-2}$ s^{-1}, respectively.

Key words resazurin; electron acceptor; glucose oxidase; glucose; resorufin; cyclic voltammetry

Resazurin (**1**) (*cf.* Chart 1) is known to act as an electron acceptor in diaphorase- or *N*-methylphenazinium methosulfate (PMS^+)-catalyzed oxidation of NAD(P)H and to be reduced to resorufin (**2**).[1—9] The reductively deoxygenated product **2** exhibits strong emission (excitation maximum at 563 nm and emission maximum at 587 nm in pH 7.4 buffer)[10,11] at wavelengths>550 nm, where potential interference in analysis of colored or turbid serum components can be avoided. Thus, the transformation of non-fluorescent **1** to fluorescent **2** has been utilized as a fluorometric indicator reaction for determination of activity[1—3] or substrates[6—8] of $NAD(P)^+$-specific dehydrogenases as well as $NAD(P)^+$ [5]: by enzymatic reaction with an $NAD(P)^+$-specific dehydrogenase, its substrate is oxidized, while $NAD(P)^+$ is transformed to NAD(P)H; **1** is reduced to **2** by thus formed NAD(P)H in the presence of diaphorase or PMS^+, depending on dehydrogenase activity, or concentration of a substrate or $NAD(P)^+$.

Based on the intriguing behavior of **1** in NAD(P)H-oxidation, it is speculated that **1** functions as an electron acceptor in enzymatic oxidation by other oxidoreductases such as glucose oxidase (GOD), being transformed to **2**. However, there have been no studies of the possibility of transformation of **1** to **2** as a fluorometric indicator reaction for enzymatic analysis coupled with enzymatic redox reactions. Recently, **2** was found to function as an electron acceptor with a color change in GOD-catalyzed oxidation of glucose under certain conditions.[12] This finding prompted us to examine how derivatives of **2** such as **1** would behave in GOD-catalyzed oxidation of glucose, and an interesting behavior of **1** in the enzymatic reaction was found. Here, we report that in GOD-catalyzed oxidation of glucose, **1** is transformed to **2** similarly to the case of diaphorase- or PMS^+-catalyzed oxidation of NAD(P)H (Chart 1), and is superior to **2** as an electron acceptor, although the observed behavior of **1** will not find direct application to enzymatic analysis of glucose unless the rate for **1** to reoxidize the reduced form of GOD can be improved.

Results and Discussion

A solution of **1** in phosphate buffer (pH 7.4, 0.1 M) had a purple color (λ_{max}, 602 nm). When a mixture of **1** (20.0 nmol), glucose (5.0 μmol), and GOD (1.0 mg) in 3.0 ml phosphate buffer (pH 7.4, 0.1 M) was incubated at 25 °C under anaerobic conditions, the mixture turned purple to fluorescent pink. In the absorption spectrum of the mixture, a new peak with λ_{max} at 571 nm appeared as soon as the incubation was started, as shown in Fig. 1. As incubation time was increased, the new peak became gradually larger, while the peak due to **1** became smaller and had almost disappeared after 15 min incubation (Fig. 1c). The new peak at 571 nm coincided with that of the deoxygenated product **2** in phosphate buffer. The color of **1** was not affected by treatment with GOD alone or with H_2O_2 under these conditions. These results suggested that the color change observed in the incubation of **1**, glucose, and GOD was due to reductive formation of **2** from **1** coupled with reoxidation of the reduced form (GOD_{red}) of GOD. The following voltammetric behavior of **1** also supported the suggestion that electron transfer to **1** from GOD_{red} yields **2**. On cyclic voltammetry of **1** in phosphate buffer, a cathodic peak at −203 mV and a couple of a redox wave (cathodic and anodic peaks at −320 and −281 mV, respectively) were observed (Fig. 2a). No anodic peak coupled with the first cathodic peak was observed on a reverse scan even when voltammetry was performed at a scan rate as high as 2.0 V/s between 0.2 and −0.25 V. The redox wave was coincident with that observed for **2** (Fig. 2b). Accordingly, it was obvious that the first cathodic peak was due to reduction of **1**, where **2** was immediately formed, to exhibit its redox wave.

This is the first report to show that **1** acts as an electron acceptor in GOD-catalyzed oxidation of glucose, being reduced to **2**, similarly to the case of diaphorase- or PMS^+-catalyzed oxidation of NAD(P)H. Previously it was found that **2** reoxidizes GOD_{red} effectively at 36 °C, but its ability becomes

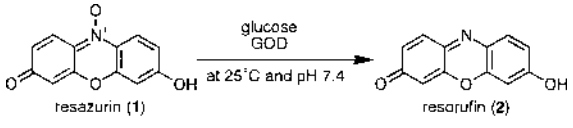

Chart 1

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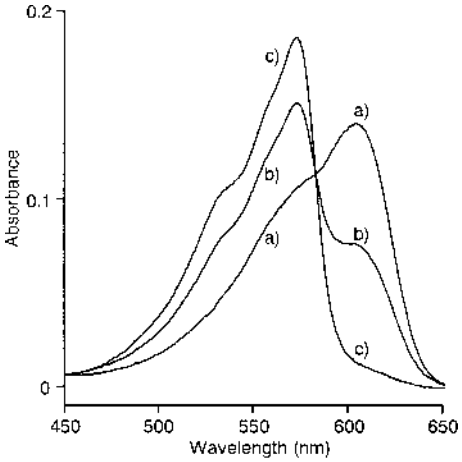

Fig. 1. Absorption Spectra Obtained Immediately (a), and after 5 min (b), or 15 min (c) Incubation of a Mixture of **1** (20.0 nmol), Glucose (5.0 μmol), and GOD (1.0 mg) in 3.0 ml Phosphate Buffer at 25 °C

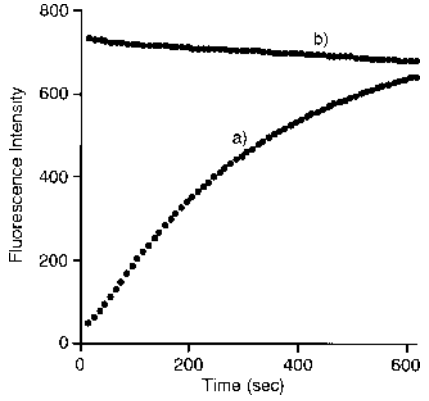

Fig. 3. Fluorometric Traces Obtained during 10 min Incubation in 3.0 ml Phosphate Buffer at 25 °C for a Mixture of **1** (10.0 nmol), Glucose, and GOD (0.5 mg) (a) and for a Mixture of **2** (10.0 nmol), Glucose, and GOD (0.5 mg) (b)

Excitation and emission wavelengths: 568 and 582 nm, respectively.

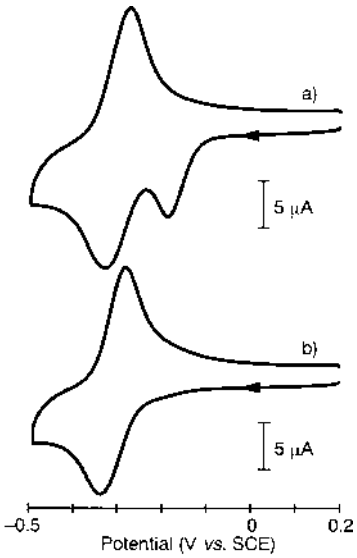

Fig. 2. Cyclic Voltammograms of **1** (0.1 mM) (a) and **2** (0.1 mM) (b) in Phosphate Buffer at Room Temperature under an N_2 Atmosphere

Scan rate: 0.1 V/s.

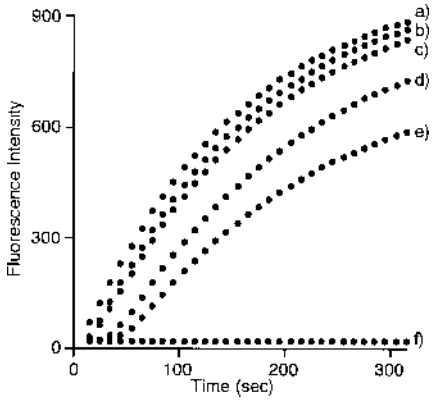

Fig. 4. Effects of Concentration of Glucose on Fluorometric Traces Obtained during 5 min Incubation of a Mixture of **1** (20.0 nmol), Glucose, and GOD (1.0 mg) in 3.0 ml Phosphate Buffer at 25 °C

Excitation and emission wavelengths: 568 and 582 nm, respectively. Concentration of glucose (μmol): 2.0 (a), 1.0 (b), 0.4 (c), 0.2 (d—f). Traces d—f were obtained on different runs under essentially the same conditions.

poor at 25 °C.[12] In Fig. 3, a fluorometric trace obtained during incubation of a mixture of **1** (10.0 nmol), glucose (5.0 μmol), and GOD (0.5 mg) in 3.0 ml phosphate buffer is compared with that of a mixture of **2** (10.0 nmol), glucose (5.0 μmol), and GOD (0.5 mg) in 3.0 ml phosphate buffer at 25 °C. During incubation for 10.0 min, a total decrease in fluorescence intensity by reaction of **2** with GOD_{red} was less than 10% of a total increase by reaction of **1** with GOD_{red}. Thus, it was demonstrated that further reduction of newly formed **2** into its dihydro derivative by GOD_{red} exerts almost no effect on the fluorescent pink coloration by the transformation of **1** to **2** through the enzymatic reaction.

Possibility of the transformation of **1** to **2** as an indicator reaction for glucose determination using only GOD was examined by a fluorometric method. Figure 4 compares fluorometric traces obtained for incubation of a mixture of **1** (20.0 nmol), GOD (1.0 mg), and glucose (2.0, 1.0, 0.4 or 0.2 μmol) in 3.0 ml phosphate buffer. The enzymatic reactions with glucose at more than 2.0 μmol resulted in fluorometric traces almost superimposed on that with 2.0 μmol glucose (Fig. 4a). No progression curves were noted when glucose at concentrations less than 0.2 μmol was subjected to the reaction. Those results are not included in Fig. 4 for simplicity. As shown in Fig. 4, changes in fluorescence intensity showed only minute dependency on glucose concentrations between 2.0 and 0.4 μmol (Figs. 4a—c). When the enzymatic reaction with 0.2 μmol glucose was repeated under essentially the same conditions, no reproducible traces were afforded (Figs. 4d—f): one of the runs gave a progression curve similar to those observed for the cases with glucose at more than 0.4 μmol, and the other did not.

These results suggested the following points: 1) the rate of reoxidation of GOD_{red} by **1** is almost saturated in the enzymatic reactions with glucose at more than 0.4 μmol in 3.0 ml phosphate buffer, and 2) in the media deoxygenated as strictly as possible, dissolved oxygen (DO) still remains in around 0.1 μmol/3.0 ml and is preferred to **1** as an electron acceptor from GOD_{red}. It was anticipated that totally anaerobic conditions could allow the present fluorometric method to work for determination of glucose at much lower concen-

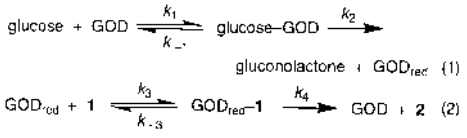

Chart 2

trations. However, attempts to realize such conditions not only by passing N_2 gas thorough the media but also by addition of sodium sulfite or ascorbic acid and ascorbate oxidase at various concentrations failed. This is because sodium sulfite and the reduced form of ascorbate oxidase rapidly reduced **1** to **2**.

Quantitative elucidation of the efficiency of **1** as an electron acceptor in GOD-catalyzed oxidation of glucose was attempted. The scheme shown in Chart 2 describes a plausible reaction sequence based on a ping-pong type mechanism. During the enzymatic reaction, a progression curve with a larger change in fluorescence intensity was observed as the concentration of **1** or GOD was increased. Taking these facts and the results in Fig. 4 into consideration, the process 1 is much faster than the process 2 when the enzymatic reaction is carried out for glucose at a high concentration such as 10.0 μmol/3.0 ml. Accordingly, the following kinetic expression for consumption of **1** was derived with a total transit time inspection at a steady state.[13)]

$$[\mathrm{GOD}]_0/v=(k_{-3}+k_4)/k_3[\mathbf{1}]k_4+1/k_4$$

where $[\mathrm{GOD}]_0$ is the initial concentration of GOD.

When the reciprocals are taken, a Michaelis–Menten type equation was derived.

$$v=k_4[\mathrm{GOD}]_0[\mathbf{1}]/\{[\mathbf{1}]+(k_{-3}+k_4)/k_3\}$$

where now the Michaelis constant (K_M) and the catalytic constant (k_{cat}) for the process 2 are $(k_{-3}+k_4)/k_3$ and k_4, respectively.

Substituting the first equation gives

$$[\mathrm{GOD}]_0/v=K_M/k_{cat}[\mathbf{1}]+1/k_{cat}$$

Thus, K_M and k_{cat} were estimated by plotting $[\mathrm{GOD}]_0/v$ against 1/[**1**]. Figure 5 shows the plot obtained for the enzymatic reactions for a mixture of **1**, GOD (0.5 mg), glucose (5.0 μmol) in 3.0 ml phosphate buffer at 25 °C. A similar linear relationship between $[\mathrm{GOD}]_0/v$ and 1/[**1**] was also observed, when the amount of GOD (0.6, 0.7, 0.8 or 1.0 mg in 3.0 ml) was changed. From slopes and intercepts on the y axis of the obtained lines, K_M and k_{cat} were estimated to be 15.0±1.3 μM and $(5.0\pm0.5)\times10^{-2}$ s^{-1}. The apparent second-order rate constant (k_{cat}/K_M) for the process 2 was then 3.3×10^3 M^{-1} s^{-1}. This value of k_{cat}/K_M is quite consistent with 3.2×10^3 M^{-1} s^{-1}, which was independently estimated in the same manner as used in the case of **2**,[12)] namely, using the assumption that the consumption of **1** obeys pseudo-first order kinetics ($v=k_{cat}/K_M\,[\mathrm{GOD}]_0[\mathbf{1}]$). The estimated k_{cat}/K_M for reaction of **2** with GOD_{red} at 36 °C was 6.6×10^2 M^{-1} s^{-1}.[12)] Thus, **1** reoxidized GOD_{red} more than 5 times faster than **2** (taking the reaction temperature into consideration), in line with the voltammetric observation that **1** was reduced at a more positive potential than **2**.

It was reported that some dyes such as methylene blue, Meldola's blue, and methylene green (k_{cat}/K_M: 1.3×10^3, 1.9×10^4, and 5.5×10^3 M^{-1} s^{-1}, respectively)[14)] acts as electron acceptors in GOD-catalyzed oxidation of glucose. However, reductive bleaching of these dyes is not likely to function as an indicator reaction in GOD-dependent colorimetry. This is not only because the ability of these dyes to act as electron acceptors is inferior to that of DO (k_{cat}/K_M: 1.0×10^6 M^{-1} s^{-1} at pH 7.4 and 25 °C)[15)] but also because reduced products of these dyes formed through reoxidation of GOD_{red} are generally susceptible to oxidative reverse reaction to the original dyes by DO. Although k_{cat}/K_M for **1** is just comparable to those for these dyes, the reduced product **2** is quite stable in the presence of GOD_{red} as well as DO at 25 °C.

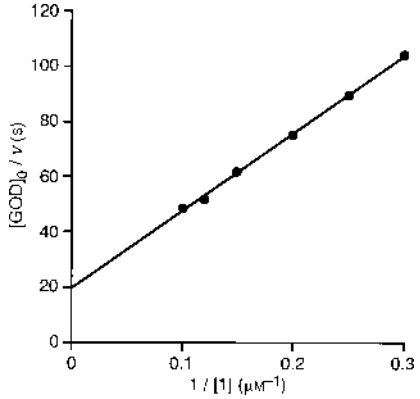

Fig. 5. Plot of $[\mathrm{GOD}]_0/v$ against 1/[**1**] for Enzymatic Reactions of **1**, Glucose (5.0 μmol), and GOD (0.5 mg) in 3.0 ml Phosphate Buffer at 25 °C

In conclusion, the non-fluorescent compound **1** was shown to act as an electron acceptor in GOD-catalyzed oxidation of glucose, being reduced to the fluorescent compound **2**. It is believed that in GOD-catalyzed oxidation of glucose, **1** acts as an electron acceptor superior to the reported dyes from the standpoint of stability of coloration process. However, the transformation of **1** to **2** as it is can not function as an indicator reaction for enzymatic analysis of glucose using GOD. In other words, practical application for glucose determination using GOD requires improvement of the rate of the fluorometric coloration in the present methodology. The most straightforward method for the improvement should be to increase the solubility of **1** by structural modification. Further studies on this point are currently underway in our laboratory.

Experimental

Reagents and Sample Solutions Resazurin sodium salt was purchased from Wako Pure Chemical Industries, Ltd. and used as **1** without further purification. GOD from Aspergillus niger (EC 1.1.3.4, 170 units/mg) and glucose were used as supplied. All solutions were prepared in phosphate buffer (0.1 M, pH 7.4; $Na_2HPO_4+NaH_2PO_4$). Glucose solutions were stored overnight to allow equilibration of α- and β-anomers. A solution of **2** was prepared from acetyl resorufin recrystallized from ethyl acetate as reported previously.[12)] All other chemicals were of reagent grade and were used without further purification. Deionized and distilled water was used throughout the present study.

Apparatus and Procedures Cyclic voltammograms were obtained with an ALS model 600 electrochemical analyzer under an N_2 atmosphere. A three-electrode configuration was employed: a glassy carbon electrode (7.07 mm^2), a saturated calomel electrode (SCE), and a platinum wire electrode as a working, a reference, and a counter electrodes, respectively. All spectrophotometric measurements were carried out under an N_2 atmosphere. Enzymatic reactions were initiated by addition of a glucose solution (1.0 ml)

to a mixture of **1** and GOD in 2.0 ml phosphate buffer in a spectrophotometric quarts cuvette (10×10×45 mm). Each of these solutions was deoxygenated by passing N_2 gas through them for more than 10 min prior to spectrophotometric measurements. Absorption spectra were obtained with a Hitachi U-3210 spectrophotometer without stirring at 25 °C. All fluorometric measurements were carried out using a JASCO Model FP-750 spectrofluorometer equipped with a JASCO ETC-272 Peltier thermostatted single cell holder with stirring at 500 rpm at 25 °C. Measurement of the fluorescence at excitation and emission wavelengths of 568 and 582 nm, respectively, was started 15 s after addition of glucose solution. To obtain v in the enzymatic reaction, changes in fluorescence intensity during 45 s after initiation were used after normalization to the concentration of **2** based on the fact that fluorescence intensity of 178.31 corresponds to 1.0 μM **2**.

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