A Kinetic Assay for Cytochrome b_5 Based on Electron Transfer to Cytochrome *c*

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Cytochrome b_5-mediated electron-transfer reaction from NADH to cytochrome *c* was exploited for the determination of cytochrome b_5. A linear relationship between cytochrome b_5 concentration and the reducing rate of cytochrome *c* was obtained in the range of 15-700 pM.

Cytochrome b_5 (cyt.b_5) is a membrane-bound hemoprotein found in many mammalian cells, in which it plays as an electron mediator in participating in a number of electron-transfer reactions.[1] For exampie, microsomal cyt.b_5 functions as a component of NADH-depending fatty acid desaturase system. In this system, cyt.b_5 accepts an electron from NADH via NADH-cyt.b_5 reductase (fp1), and then supplies it to various desaturases. Additionally, it is well known that cyt.b_5 also supplies an electron to externally added cytochrome *c* (cyt.*c*). This unphysiological reaction has been widely investigated as a model of interprotein electron transfer.[2,3] On the other hand, the determination of cyt.b_5 is usually carried out spectrophotometrically by measuring absorbance of heme.[4] However, this method requires a relatively large amount of materials, and can not determine cyt.b_5 in a nano- or picomolar level. In this study, we have developed a high sensitive detection method for cyt.b_5 using NADH-cyt.*c* reductase system.

Figure 1 shows NADH-cyt.*c* reductase system.[5] This system could be assumed to be a semi-enzymatic cycling system, in which oxidized form of cyt.b_5 is enzymatically reduced by NADH to produce reduced form of cyt.b_5 (cyt.b_5*) and NAD^+, and then, cyt.b_5* is non-enzymatically oxidized by cyt.*c* to form cyt.b_5 and reduced form of cyt.*c* (cyt.*c**). Cytochrome *c** is accumulated with each turn of the cyt.b_5 redox cycling until NADH or oxidized form of cyt.*c* is completely consumed. In this system, we have found that the reduction rate of cyt.*c* is dependent on cyt.b_5 concentration. Thus, the kinetic assay for cyt.b_5 was developed by measuring spectrophotometrically the formation rate of cyt.*c** as an indicator.

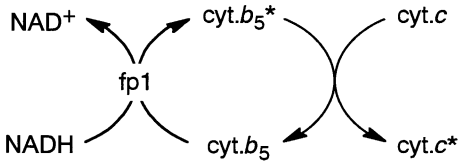

Figure 1. NADH-cyt.*c* reductase system. Asterisk represents reduced form.

Cytochrome b_5 and fp1 were purified from pig liver microsomes by using the methods of Kamataki[6] and Iyanagi,[7] respectively. The concentration of cyt.b_5 in a stock solution was determined spectrophotometrically from the difference spectrum between reduced and oxidized forms, for which a $\Delta\varepsilon$(424-409 nm) value of 181 mM^{-1} cm^{-1} was used.[4] NADH and horse heart cyt.*c* were obtained from Wako Pure Chemicals.

The reduction rate of cyt.*c* was measured as follows: A 1-cm glass cell was placed on a thermostated cell holder in a spectrophotometer, into which a pH buffer solution of cyt.*c* was added. Then, a mixed solution of NADH, fp1, and cyt.b_5 was added to start the reaction, the final volume being 2 ml. The time course of the reaction was followed by measuring the increase in Δabsorbance between cyt.*c** and cyt.*c* at 550 nm.[8]

First, the time course of cyt.*c* reduction was monitored with various concentrations of cyt.b_5. As shown in Figure 2, a linear increase in the Δabsorbance was observed at each concentration of cyt.b_5. The reduction rate of cyt.*c* was increased with increasing cyt.b_5 concentration. Additionally, in the absence of cyt.b_5, no appreciable reduction of cyt.*c* was observed. These results indicate that the NADH-cyt.*c* reductase system could be applied to the determination of cyt.b_5. In this study, Δabsorbance after 1 min is referred to as the activity of NADH-cyt.*c* reductase system.

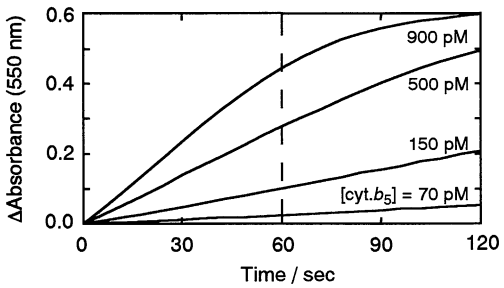

Figure 2. Time course of the reduction of cyt.*c* in the presence of various concentration of cyt.b_5. 0.10 mM NADH, 0.1 unit ml^{-1} fp1, 50 μM cyt.*c*, 50 mM potassium phosphate, pH 7.5, 35 °C.

The effect of the reaction temperature on the activity was examined in the range of 15 to 40 °C. The activity increased with an increase in temperature to a maximal value at 35 °C, after which it decreased. Thus, the optimum temperature for the reaction was determined to be 35 °C. The influence of pH was tested over the range of pH 6 to 8. The activity approached a maximum at pH 7.5, and then decreased with increasing pH. Thus, pH 7.5 was chosen for the recommended condition.

Generally, in the enzymatic cycling system including two enzymes,[9] the rate of the overall reaction (V) in the steady state is written as:

$$V = k\,C \qquad 1$$

with an overall rate constant (k):

$$k = k_1\,k_2 / (k_1 + k_2) \qquad 2$$

where C is the total concentration of a recycling substrate; k_1 and k_2 are the first-order rate constants for the first and second enzymatic reactions, respectively, and are dependent on the concentration of each enzyme. From equation 1, the reaction rate is proportional to the recycling substrate concentration with a slope of k. Thus, the system can be used for an assay of the recycling substrate by measuring the reaction rate, V, at fixed enzyme concentrations. On the other hand, the present system is not bi-enzymatic reaction, but the kinetics mentioned above could be applied to the present system, in which the first reaction is fp1-catalyzed electron transfer from NADH to cyt.b_5, and the second reaction is non-enzymatic electron transfer from cyt.b_5 to cyt.c. In order to allow the first and second reactions to proceed in the first-order conditions, the concentrations of two substrates, NADH and cyt.c in the present system, need to be much greater than that of the recycling substrate, cyt.b_5. Additionally, in the present system, the second reaction proceeds non-enzymatically, so that k_2 could be constant. From equation 2, on increasing k_1, k is increased, and then maintained constant at k_2 when k_1 is much greater than k_2. Consequently, the concentration of fp1, on which k_1 is dependent, could directly affect to the overall reaction rate.

The concentrations of the two substrates, NADH and cyt.c, and one enzyme, fp1, in the system were thus optimized. The effects of NADH and cyt.c concentrations on the activity were investigated in the range of 0.05 to 0.3 mM and 10 to 100 μM, respectively. The activity increased with an increase in NADH concentration, and then reached plateau in the range of 0.1 to 0.2 mM, after which it decreased. On the other hand, the activity also increased with increasing cyt.c concentration, and then kept constant beyond 30 μM. The plateaus in the activity indicate that these concentration ranges of NADH and cyt.c are high enough to remain constant during the reaction time. Under these conditions, the rates of the two reactions in the present system should be only dependent on the concentration of cyt.b_5 at a fixed fp1 concentration. The optimum NADH and cyt.c concentrations were, thus, determined to be 0.1 mM and 50 μM, respectively.

The effect of the fp1 concentration on the activity was also investigated. Unfortunately, a stock solution of fp1 was too dilute to determine the fp1 concentration. Thus, we used the fp1 activity for the reduction of ferricyanide to ferrocyanide[5] ranging from 0 to 1.0 unit ml^{-1} instead of the concentration. As a result, it was shown that the reduction rate of cyt.c increased linearly with increasing the fp1 activity. This means an increase in the overall rate constant, k, with increasing k_1, as mentioned above. Additionally, from a linear increase in the reduction rate it is expected that k_1 would be smaller than k_2 over the fp1 activity range studied. The fp1 activity was chosen to be 0.1 unit ml^{-1} for the recommended condition in this study.

Under these optimal conditions, a small quantity of cyt.b_5 could produce a large quantity of cyt.c (and NAD^+), thus the system could effectively act as a chemical amplifier in the quantification of cyt.b_5 by measuring reduction of cyt.c. Analytical calibration curve for cyt.b_5 was prepared under the optimal conditions. Logarithmic calibration curve is shown in Figure 3. The calibration curve appeared to be linear over the concentration range from the detection limit of 15 to 700 pM of cyt.b_5. Above 700 pM of cyt.b_5, the reduction of cyt.c was too fast to give a linear increment in the Δabsorbance in the first 1 min (Figure 2), indicating the reaction proceeds no longer in the first-order conditions. The detection limit for cyt.b_5 was defined as the concentration yielding the Δabsorbance of 0.008, which is 4 times greater than the accuracy in the absorbance of the spectrophotometer used. The detection limit of the present method for cyt.b_5 was a factor of 3500 times better than that of the conventional spectrophotometric method.[4] The relative standard deviation of the activity in five successive experiments was 5.3% at 150 pM of cyt.b_5.

The detection limit can be much lowered by extending the measurement time of cyt.c reduction and/or by using much higher activity of fp1. From the calibration curve, the first-order rate constant in this reaction, k can be estimated to be about 400 s^{-1}, which is the number of redox cycles of cyt.b_5 per second, and is much greater than that found in other enzymatic cycling systems.[9] This should be ascribed to the fast reaction of cyt.b_5 with cyt.c. The rate constant for electron transfer from cyt.b_5 to cyt.c including cyt.b_5-cyt.c complex formation and dissociation, and structural change in the complex was reported to be 1600 s^{-1} or more.[3] Therefore, in the present system, the electron transfer from NADH to cyt.b_5 via fp1 should be rate-limiting reaction under these conditions as expected above.

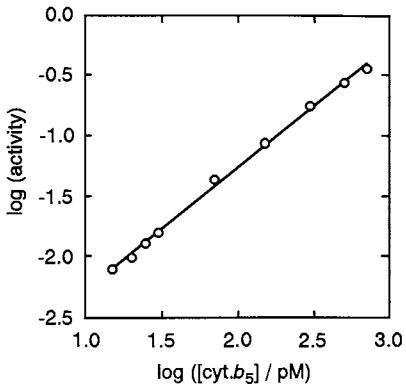

Figure 3. Calibration curve for cyt.b_5. The conditions are the same as in Figure 2.

References and Notes

1 N. Oshino, *Pharmacol. Ther. A*, **2**, 477 (1978).
2 A. G. Mauk, M. R. Mauk, G. R. Moore, and S. H. Northrup, *J. Bioenerg. Biomembr.*, **27**, 311 (1995).
3 B. Durham, J. L. Fairris, M. McLean, F. Millett, J. R. Scott, S. G. Sligar, and A. Willie, *J. Bioenerg. Biomembr.*, **27**, 331 (1995).
4 T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
5 K. Mihara and R. Sato, *J. Biochem.*, **71**, 725 (1972).
6 T. Kamataki, K. Maeda, Y. Yamazoe, T. Nagai, and R. Kato, *Biochem. Biophys. Res. Commun.*, **103**, 1 (1981).
7 T. Iyanagi, *Biochemistry*, **16**, 2725 (1977).
8 B. F. van Gelder and E. C. Slater, *Biochim. Biophys. Acta*, **58**, 593 (1962).
9 E. Valero and F. García-Carmona, *Anal. Biochem.*, **239**, 47 (1996).