Two Phases of Ferricyanide Reductase Activity in Ehrlich Cell Plasma Membranes

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Ehrlich cell plasma membrane vesicles have a ferricyanide reductase activity that shows two phases. These two phases were kinetically characterized. Evidence is presented for a differential effect of trypsin on both phases.

Eukaryotic plasma membrane redox systems have been studied in perfused organs, tissues, isolated cells, and native vesicles [1, 2 and references therein]. We have previously studied the plasma membrane ferricyanide reductase activity in intact Ehrlich cells [3]. This activity had been first described by Kilberg and Christensen in native plasma membrane vesicles [4]. The existence of a fast and a slow rate of plasma membrane redox activity has been previously described in intact cells [5, 6]. In our previous report on ferricyanide reductase activity in intact Ehrlich cells, we could not show direct evidence for these two phases because of the end-point method used [3]; however, the data in Table I in that paper show a decrease in the velocity of ferricyanide reduction with increasing incubation times. It seems that these data also suggest the existence of the two phases. For this reason, we decided to revisite the kinetic behaviour of ferricyanide reductase activity in Ehrlich cell plasma membrane vesicles.

Highly pure plasma membrane vesicles were isolated from Ehrlich cells by two-phase compartmentation in polyethyleneglycol/dextran, as described elsewhere [7]. The purity of the plasma membrane obtained was greater than 95%, as determined by marker enzyme assays. Ferricyanide reduction and NADH oxidation were kinetically followed in a Shimadzu UV-160 spectrophotometer at 420 and 340 nm, respectively. The assay medium contained 0.5 mM ferricyanide, 0.5 mM NADH and plasma membrane vesicles (60–80 µg protein) in phosphate buffered saline (PBS: 154 mM NaCl, 7 mM KCl, 11 mM sodium phosphate, pH 7.4) or 5 mM Hepes, pH 7.4, at 1 ml final volume. To study the effect of pH, different buffered media at 5 mM final concentration were used: acetate (pH 5), phosphate (pH 6, 6.8, and 7.4), Tris (pH 8, 8.5, and 9), and carbonate (pH 10). In the experiments with trypsin, plasma membrane vesicles (1 mg/ml) were preincubated for 15 min at 37 °C in the absence or presence of 0.1% (w/v) trypsin; thereafter, an excess of trypsin inhibitor was added (1 mg trypsin inhibitor/mg trypsin) and reductase activity was determined. Proteins were determined as described by Bradford [8].

When highly pure plasma membrane vesicles from Ehrlich cells were used to assay NADH : ferricyanide oxidoreductase activity in PBS, two phases, one fast and transient and the other one slow, were observed in both NADH oxidation and FCN reduction (Fig. 1). When the assay was carried out in 5 mM Hepes, pH 7.4 both phases were undistinguishable, apparently due to partial inhibition of the fast phase activity. Since Hepes buffer differed from PBS in both ionic strength and osmolarity, we tested the role of these two parameters on redox activity. Table I shows that the use of a medium with high osmolarity and low ionic strength (5 mM Hepes, 0.4 M sucrose, pH 7.4) permited us to discriminate between fast and slow phases. Substitution of 0.4 M sucrose by 0.2 M NaCl resulted in a significant increase in both phases. These data are in agreement with those of Sun *et al.* [5], which show an increase in ferricya-

Table I. Effect of osmolarity and ionic strength on ferricyanide reductase activity of plasma membrane vesicles isolated from Ehrlich cells. The rate of 0.4 mM ferricyanide reduction in the presence of 0.1 mM NADH was determined in 0.4 M sucrose, 5 mM Hepes, pH 7.4 (control) or in isoosmolar 0.2 M NaCl, 5 mM Hepes, pH 7.4. Data are given as percentages of activity and represent the means (± S.D.) of three different determinations.

Condition	% Activity Rapid phase	% Activity Slow phase
Control	100 ± 2	100 ± 5
0.2 M NaCl	124 ± 1*	125 ± 1*

* Significant as compared with control values ($p < 0.01$).

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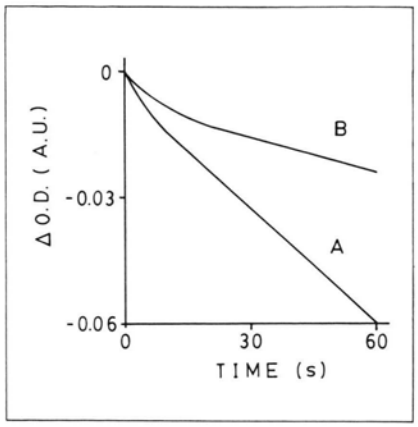

Fig. 1. Time course of NADH-ferricyanide oxidoreductase activity in plasma membrane vesicles isolated from Ehrlich cells. The activity was determined by following ferricyanide (0.5 mM) reduction at 420 nm (A) or NADH (0.5 mM) oxidation at 340 nm (B) by plasma membranes (60–80 μg protein) in phosphate buffered saline. The two clear phases shown under these conditions could not be distinguished in a low osmolarity buffer.

nide reductase activity with increasing NaCl concentrations, using a medium with 0.25 M sucrose as a control. This effect does not seem to be due to an increase in ionic strength but rather seems to be specific for sodium ions. This effect of sodium ions could reflect the narrow interrelationship between plasma membrane redox system and Na^+/H^+ antiporter [9, 10]. Kilberg and Christensen [4] did not detect these two phases when they assayed the redox activity in 50 mM Tris, pH 7.4, 0.5 mM EGTA, 3 mM $MgCl_2$, a medium with relatively low osmolarity. Since PBS is closer to physiological conditions than 5 mM Hepes buffer or the medium used by Kilberg and Christensen [4], the appearance of two phases could reflect the physiological behaviour of the system.

The kinetic analysis of both fast and slow phases for ferricyanide reduction revealed saturation kinetics with K_m values of 13, and 41 μM, and V_{max} values of 874 and 250 nmol/mg protein/min, respectively. The apparent affinity for ferricyanide was higher in both phases than that reported by Kilberg and Christensen [4]. In contrast with data shown by these authors, inhibition by ferricyanide was not observed during the slow phase at concentrations up to 1 mM; nonetheless, during the fast phase there was inhibition by ferricyanide at concentrations above 0.5 mM. The redox system presented very high affinities for NADH during both the fast and the slow phases: 2.5 and 9 μM, respectively. These values are lower than the apparent K_m for NADH in the ferricyanide reduction reported by Kilberg and Christensen [4].

The three sulfhydryl reagents assayed (mersalyl, PCMBS, and NEM) produced a significant inhibition of the plasma membrane redox activity; this inhibitory effect was similar for both phases. Maximum inhibition (70–80%) was obtained with mersalyl. The inhibition produced by PCMBS (60%) was less than that previously reported for PCMB [4]; this difference could be explained by the greater penetrability of PCMB through biological membranes.

Ferricyanide reductase remained active at acidic pH values. Activity had a maximum in the wide pH range between 5 and 7.4 (results not shown). However, the activity dropped at basic pH values (50% of maximum activity at pH 9). Since the global oxidoreduction reaction is:

$$NADH + H^+ + 2\ \text{ferricyanide} \rightleftharpoons 2\ \text{ferrocyanide} + NAD^+ + 2H^+,$$

it is clear that the reduction of ferricyanide produces a release of protons; for this reason, taking into account the Le Chatelier principle, it should be expected that the reaction was favoured by basic pH values. However, the observed effect was just the contrary; this fact might indicate the presence of histidine in the active center.

Ferricyanide reductase of plasma membrane vesicles isolated from Ehrlich cells is very sensitive to trypsin proteolysis (Table II). The inhibitory effect was more pronounced during the slow phase: a 15 min preincubation with exogenously

Table II. Effect of trypsin treatment on ferricyanide reductase activity. Plasma membrane vesicles (1 mg/ml) were preincubated for 15 min at 37 °C in the absence or presence of 0.1% (w/v) trypsin. Afterwards, an excess of trypsin inhibitor (1 mg of inhibitor per mg of trypsin) was added, and ferricyanide reductase activity was determined as described in Material and Methods. Data are given as percentage of activity and represent the means (± S.D.) of three different experiments.

	Rapid phase (% of activity)	Slow phase (% of activity)
Control	100 ± 4	100 ± 7
+ Trypsin	43 ± 15*	15 ± 1*

* Significant as compared with control values ($p<0.01$).

added 0.1% (w/v) trypsin produced 85% inhibition in ferricyanide slow reduction rate, but only 57% inhibition in ferricyanide fast reduction rate. This difference in susceptibility to trypsin could suggest the functioning of at least two different isozymes. In fact, when solubilized plasma membrane proteins were separated by native electrophoresis, two bands of activity were detected by *in situ* dyeing (unpublished result). When exogenous trypsin is added, the protease is able to digest only outer extrinsic proteins and outer hydrophilic domains of transplasma membrane proteins. The pronounced inhibition of redox activity observed after trypsin treatment suggests that such hydrophilic domains are important for the ferricyanide reductase activity. Sun *et al.* [11] have reported almost total inhibition of NADH-diferric transferrin reductase and NADH-ferricyanide reductase activities. However, NADH-ascorbate free radical reductase of rat liver plasma membrane is less sensitive to trypsin treatment [12].

In conclusion, the occurrence of two phases in plasma membrane redox activity seems to be a common behaviour [5, 6, this paper], whose actual physiological significance and relevance remain to be elucidated. Whether or not these two phases are the result of the superposition of two activities should be determined after purification of the active plasma membrane redox systems and their reconstitution in artificial liposomes.

[1] F. L. Crane, I. L. Sun, M. G. Clark, C. Grebing, and H. Löw, Biochim. Biophys. Acta **811,** 233–264 (1985).
[2] F. L. Crane, I. L. Sun, R. Barr, and H. Löw, J. Bioenerg. Biomembranes **23,** 773–803 (1991).
[3] M. A. Medina, F. Sánchez-Jiménez, J. A. Segura, and I. Núñez de Castro, Biochim. Biophys. Acta **946,** 1–4 (1988).
[4] M. S. Kilberg and H. N. Christensen, Biochemistry **18,** 1525–1530 (1979).
[5] I. L. Sun, F. L. Crane, and J. Y. Chou, Biochim. Biophys. Acta **886,** 327–336.
[6] H. Löw, F. L. Crane, C. Grebing, M. Isaksson, A. Lindgren, and I. L. Sun, J. Bioenerg. Biomembranes **23,** 903–917 (1991).
[7] P. Luque, J. Márquez, I. Núñez de Castro, and M. A. Medina, J. Membrane Biol. **123,** 247–254 (1991).
[8] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[9] R. García-Cañero, in: Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport. I. Animals (F. L. Crane, D. J. Morré, and H. Löw, eds.), pp. 237–246, CRC Press, Boca Ratón 1990.
[10] M. A. Medina and I. Núñez de Castro, in: Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport. I. Animals (F. L. Crane, D. J. Morré, and H. Löw, eds.), pp. 247–257, CRC Press, Boca Ratón 1990.
[11] I. L. Sun, P. Navas, F. L. Crane, D. J. Morré, and H. Löw, J. Biol. Chem. **262,** 15915–15921 (1987).
[12] P. Navas, A. Estévez, M. I. Burón, J. M. Villalba, and F. L. Crane, Biochem. Biophys. Res. Commun. **154,** 1029–1033 (1988).