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# Purification of an NADPH-dependent diaphorase from membrane of DMSO-induced differentiated human promyelocytic leukemia HL-60 cells

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**Abstract NADPH diaphorase activity was found in membrane of DMSO-induced differentiated human promyelocytic leukemia HL-60 cells. This membrane-bound diaphorase activity increased dramatically during differentiation of HL-60 cells. A dye reductase was extracted from membrane of DMSO-induced differentiated HL-60 cells with *n*-octyl glucoside and sodium cholate in the presence of several protease inhibitors such as PMSF, DIFP, TLCK, antipain, chymostatin, leupeptin, pepstatin A and trypsin inhibitor. The NADPH diaphorase was highly purified by two-stage sequential column chromatographies. The purified enzyme, showing both SOD-insensitive cytochrome *c* and NBT reductase activities, migrated with an apparent molecular mass of 77 kDa on SDS-PAGE. When the purification of this diaphorase was carried out in the presence of only three protease inhibitors, PMSF, DIFP and TLCK, a partially proteolyzed form of the diaphorase with a molecular mass of 68 kDa was prepared. The proteolyzed diaphorase exhibited only an NADPH-dependent cytochrome *c* reductase. The NADPH diaphorase gave a positive cross-reaction to polyclonal antibodies raised against microsomal NADPH-cytochrome P450 reductase from rabbit liver.**

*Key words:* NADPH-dependent diaphorase; Cytochrome *P*-450 reductase; HL-60 cell; Neutrophil

## 1. Introduction

Plasma membrane dehydrogenases represent a poorly defined group of oxidoreductases that have been demonstrated in all cells tested [1] but their real functions have not been established so far. Several investigators reported that some diaphorases and NADPH-binding proteins associated with the NADPH oxidase system which functions to produce superoxide anion that contributes to microbicidal function [2]. Laporte et al. [3] reported that an NADPH-dependent membrane-bound flavoprotein dehydrogenase (NADPH-dependent cytochrome *c* reductase) was purified from rabbit peritoneal neutrophils. This purified enzyme contained FAD and FMN (1/1) as a prosthetic group and migrated with a molecular mass of 77 kDa on SDS-PAGE. This enzyme was able to enhance the rate of formation of superoxide anion in a cell-free system of NADPH oxidase. Miki et al. [4] also showed that an NADPH-dependent NBT reductase, which was partially purified from solubilized membrane of porcine neutrophils, was an essential component of superoxide-generating NADPH oxidase. Ge et al. [5] proposed that the arylazido-β-alanyl-[$^{32}$P]NADPH-labeled protein with a molecular mass of 52 kDa is only an NADPH-binding cytosol component of the neutrophilic superoxide-generating oxidase in a cell-free system, and suggested that the NADPH-binding protein might be a flavoprotein containing FAD. In a more recent study, Cross et al. [6] suggested that many diaphorase activities associated with NADPH oxidase, which were reported in many investigations, seemed likely to be artifacts due either to superoxide anion-mediated reduction of dye acceptors or to 'non-oxidase related' diaphorase activities. They found that membrane-associated cytochrome $b_{558}$ by itself showed NADPH diaphorase activity only in an absolute reconstitution of NADPH-oxidase under anaerobic conditions, and using Indonitrotetrazolium violet as an electron acceptor.

In previous studies we have described the presence of an NADPH diaphorase that contained FAD and FMN (1/1) as a prosthetic group in human neutrophils and differentiated HL-60 cells, and reported its characteristics [7–10]. We have also purified an NADPH-dependent cytochrome *c* reductase from the cytosolic fraction of HL-60 cells with a molecular mass of 68 kDa, and suggested that the enzyme might be released by limited proteolysis of the membrane-bound native form during its purification [7]. The isolation of the membrane-bound diaphorase to electrophoretic homogeneity was extremely difficult due to very low content and instability after solubilization from the membrane. In the present study we were successful in preparing a highly purified intact NADPH diaphorase from the membrane fraction of differentiated HL-60 cells in the presence of protease inhibitors, and then its properties were compared with those of NADPH-dependent dehydrogenases reported so far.

## 2. Materials and methods

### 2.1. Materials

Chemicals, enzymes and antibodies were obtained from the following companies. Sigma: ferricytochrome *c* (horse heart, type VI), NBT, sodium cholate, DIFP, PMSF, TLCK, antipain, chymostatin, leupeptin, pepstatin A, trypsin inhibitor (soybean), NADH, NADPH; Bio-Rad: pre-stained molecular makers for SDS-PAGE; Pharmacia: 2′,5′-ADP-Sepharose 4B, DEAE-Sepharose CL-6B; Oxford Biomedical Research Inc.: polyclonal goat IgG anti-rabbit liver microsomal cytochrome P450 reductase; Dojin Chemicals (Japan): OG; Wako Pure Chemicals (Japan): SOD and other chemicals.

### 2.2. Isolation of the DMSO-induced differentiated HL-60 cells

Human promyelocytic leukemia HL-60 cells, kindly provided by Dr. M. Yamada (Faculty of Science, Yokohama City University), were

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*Abbreviations:* P450 reductase, microsomal NADPH-cytochrome *P*-450 reductase from rabbit liver; DMSO, dimethyl sulfoxide; DIFP, diisopropyl fluorophosphate; NBT, Nitroblue tetrazolium; OG, *n*-octyl β-D-glucopyranoside; PAGE, polyacrylamide gel-electrophoresis; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; NADPH diaphorase, NADPH-dependent diaphorase.


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grown in suspension in 55 $cm^2$ Falcon tissue culture dishes containing 20 ml of RPMI 1640 (Gibco BRL) supplemented with 10 mM HEPES (pH 7.4), 10% heat-inactivated fetal calf serum and kanamycin (50 μg/ml) at 37°C in a humidified incubation with 5% $CO_2$. HL-60 cells were grown in 300 tissue culture dishes at $2.5 \times 10^5$ cells/ml, and differentiation was induced by addition of 1.25% DMSO for 10 days [11]. Differentiated HL-60 cells were harvested by centrifugation and washed 3 times with 100 ml of PBS. After centrifugation, the volume of packed wet cells was about 10 ml.

*2.3. Preparation of subcellular fractions and extraction of membrane proteins*

The cells were suspended in cell relaxation buffer (5 times the volume of packed wet cells) at 4°C for 15 min and disrupted in the same buffer by the nitrogen decompression method at a pressure of 800 psi for 20 min with repeated agitation. The disrupted cells were centrifuged at $500 \times g$ for 5 min to remove nuclei and unbroken cells. The supernatant was loaded onto 20, 42 and 70% sucrose gradients and centrifuged at $72,000 \times g$ for 1 h. The cloudy membrane fraction at the interface between the 20 and 42% sucrose gradients was collected. The membrane fraction was diluted with 4 times the volume of PBS containing 0.5 mM PMSF, 50 μM DIFP, 1 μg/ml TLCK, and the protease inhibitor cocktail. After centrifugation at $13,000 \times g$ for 10 min, the precipitate was collected and washed 2 times by suspension with PBS containing protease inhibitors. This precipitated fraction was used as a membrane fraction. The precipitate was suspended again in 5 ml of the solubilization buffer, and proteins were extracted with 30 mM OG and 0.5% sodium cholate at 4°C for 8 h under gentle agitation. The solubilized membrane was centrifuged at $105,000 \times g$ for 1 h and the supernatant was used for further purification of the diaphorase. After centrifugation of a differential sucrose gradient, the supernatant was collected and centrifuged again at $105,000 \times g$ for 1 h. This supernatant was used as a cytosol fraction. A PMA-stimulated membrane was prepared as follows: cells were incubated in the cell relaxation buffer containing 1 μM PMA at 36°C for 10 min and the membrane was separated by the above-mentioned procedure.

*2.4. Buffers*

(i) Protease inhibitor cocktail: 10 μg/ml of antipain, chymostatin, leupeptin, pepstatin A and trypsin inhibitor (soybean). (ii) Cell relaxation buffer: 2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM $NaHCO_3$ (pH 7.8), containing 0.5 mM PMSF, 50 μM DIFP, 1 μg/ml TLCK, and protease inhibitor cocktail. (iii) 20, 42 and 70% sucrose solutions: each solution contained 2 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5 mM PMSF, 50 μM DIFP, 1 μg/ml TLCK, and protease inhibitor cocktail. (iv) Solubilization buffer: 50 mM HEPES (pH 7.5), containing 1 mM EDTA, 10% glycerol, 30 mM OG, 0.5% sodium cholate, 0.5 mM PMSF, 50 μM DIFP, 1 μg/ml TLCK, and protease inhibitor cocktail. (v) Buffer A: 50 mM HEPES (pH 7.5), containing 1 mM EDTA, 10% glycerol, 10 mM OG, 0.1 mM PMSF, 10 μM DIFP and 1 μg/ml TLCK.

*2.5. Polyacrylamide gel-electrophoresis and enzyme staining for activity*

SDS-PAGE (10% gel) was carried out according to the method of Rudolph and Krueger [12]. Following electrophoresis, proteins on the gel were stained with silver staining kit (2D-silver stain II; Daiichi Pure Chemical Co.). Non-denaturing PAGE (7.5% gel) was performed in the presence of 35 mM OG at 3°C according to the previously described method [9]. After non-denaturing electrophoresis, the gel was cut into two fragments, one of which was used for protein staining with Coomassie brilliant blue R-250 and the other for visualization of an NADPH-dependent NBT reductase in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.4 mM NADPH, 0.5 mM NBT, 8 mM $MgCl_2$ and 10% glycerol at room temperature.

*2.6. Western blotting analysis*

Proteins separated by SDS-PAGE (10% gel) were transferred to a polyvinylidene difluoride membrane (Immobilon PVDF membrane; Millipore) according to the method of Towbin et al. [13]. The blotted PVDF membrane was incubated in 4% skim milk in PBS at room temperature for 2 h. After the washing process with PBS containing 0.1% Tween 20, the PVDF membrane was exposed first to anti-rabbit liver microsomal cytochrome P450 reductase goat IgG (about 20 μg) overnight at 4°C and then to horseradish peroxidase-linked anti-goat IgG rabbit IgG (1:5000 dilution) for 1 h at room temperature. Antibodies were diluted with PBS containing 0.1% Tween 20 and 4% skim milk. The PVDF membrane was washed thoroughly with PBS containing 0.1% Tween 20. The Western blots were visualized with 3,3′-diaminobenzidine tetrahydrochloride in PBS containing 0.03% $H_2O_2$.

*2.7. Determination of diaphorase activities*

NADPH diaphorase activities were determined spectrophotometrically at 36°C in 35 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM NADPH (or 0.2 mM NADH), 8 mM $MgCl_2$ and 0.1 mM dye electron acceptors. The reaction was started with the addition of NADPH or NADH. Ferricytochrome *c* and NBT were used as dye electron acceptors. Cytochrome *c* and NBT reductase activities were measured by an increase in absorbance of ferrocytochrome *c* at 550 nm and NBT-formazan at 540 nm, respectively, and quantified by using an absorbance coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ for ferrocytochrome *c* [14] and 7.2 $mM^{-1} \cdot cm^{-1}$ for NBT-formazan [15], respectively. The dye reduction with superoxide anion was eliminated by addition of 1,750 units SOD per ml of reaction mixture.

*2.8. Purification of NADPH diaphorase*

The solubilized membrane fraction from differentiated HL-60 cells, the supernatant at $105,00 \times g$ centrifugation, was diluted with 3 times its volume of buffer A without 10 mM OG and applied to a DEAD-Sepharose CL-6B column ($1.6 \times 5$ cm) equilibrated with buffer A. The column was washed successively with buffer A (4 times the volume of gel) and buffer A containing 0.1 M NaCl (4 times the volume of gel). An NADPH diaphorase was eluted with buffer A containing 0.5 M NaCl (the same volume of gel). The 0.5 M NaCl eluate was diluted with 5 times its volume of buffer A and applied immediately to a 2′,5′-ADP-Sepharose 4B column ($1 \times 2.5$ cm) equilibrated with buffer A. The affinity column was washed successively with buffer A (100 ml), buffer A containing 0.5 M NaCl (50 ml) and again with buffer A (50 ml). An NADPH diaphorase was eluted with buffer A containing 1 mM NADPH (3.5 ml). NADPH in the eluate was removed by dialysis against buffer A. Protein was determined by the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as a standard.

## 3. Results

*3.1. An increase in NADPH diaphorase activity during DMSO-induced granulocytic differentiation of HL-60 cells*

Granulocytic differentiation of human promyelocytic leukemia HL-60 cells was induced by the addition of 1.25% DMSO for 10 days. The differentiated HL-60 cells were gently disrupted by cavitation, and separated into cytosol and membrane fractions by differential centrifugation in the presence of several protease inhibitors. SOD-insensitive NADPH diaphorase activities, independent of the reduction by superoxide anion, dramatically increased in the membrane fraction of the differentiated HL-60 cells (Fig. 1), although the increase was less pronounced in the cytosol fraction during cell differentiation.

The activities of NADPH-dependent NBT reductase in the solubilized membrane fractions of differentiated and undifferentiated HL-60 cells were analyzed by non-denaturing-PAGE (Fig. 2). A pronounced band, which was strongly stained by an NADPH-dependent NBT reductase activity, was visualized around a molecular mass of 80 kDa, and a faint band was observed around 140 kDa. The activity of an NADPH-dependent NBT reductase was strongly revealed in the membrane fraction of differentiated HL-60 cells in contrast to that of undifferentiated cells (Fig. 2B; lane 1, undifferentiated membrane and lane 3, differentiated membrane). Then, we studied whether the NADPH-dependent NBT reductase activities were affected by PMA stimulation that activated superoxide-generating NADPH oxidase. The membrane-bound NADPH-dependent NBT reductase activities of both undifferentiated and differentiated HL-60 cells showed little change under PMA

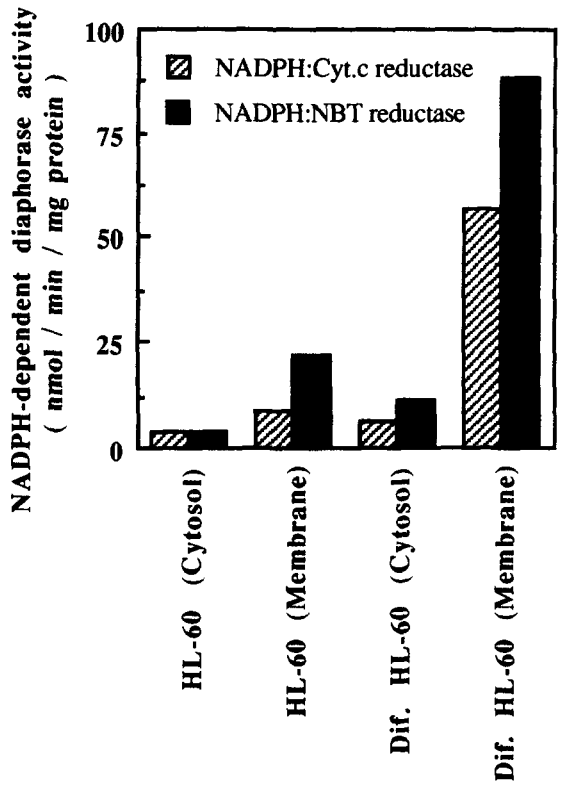


Fig. 1. Induction of NADPH diaphorase activities in subcellular fractions during DMSO-induced granulocytic differentiation of HL-60 cells. Cells were disrupted in the presence of PMSF, DIFP, TLCK, and protease inhibitor cocktail, and separated into subcellular fractions by a differential centrifugation. The NADPH diaphorase activities were measured in the presence of SOD.

stimulation (Fig. 2B; labe 2, undifferentiated membrane with PMA stimulation and lane 4, differentiated membrane with PMA stimulation).

These results strongly suggest that the membrane-bound NADPH diaphorase is induced during differentiation of HL-60 cells, and that the diaphorase activities are independent from superoxide-generating NADPH oxidase.

### 3.2. *Purification of NADPH diaphorase from the membrane fraction*

The dye reductase, assayed as a catalyst of electron transfer from NADPH to cytochrome *c* and NBT, was purified from the unstimulated membrane of differentiated HL-60 cells. The membrane fraction was separated by a centrifugation of differential sucrose gradient, and membrane-bound proteins were extracted with 30 mM OG and 0.5% sodium cholate. The enzyme was successfully purified with two-stage sequential column chromatographies, DEAE-Sepharose CL-6B and 2′,5′-ADP-Sepharose 4B, without any process of concentration or dialysis. The results of the purification are summarized in Table 1. Based on the NADPH-dependent cytochrome *c* reductase activity, this method yielded an overall purification of 89-fold from the membrane fraction with a 7.4% yield (specific activity, 4.99 μmol of ferrocytochrome *c*/min per mg of protein). This purified enzyme coincidentally showed an NADPH-dependent NBT reductase activity (specific activity, 6.40 μmol of NBT-formazan/min per mg of protein). Overall purification and recovery for NADPH-dependent cytochrome *c* reductase were nearly in parallel with those for NADPH-dependent NBT reductase (Table 1). This diaphorase could not catalyze the electron transfer from NADH to cytochrome *c* and NBT. The purified enzymes were stable in buffer A after storage at −70°C for a month.

### 3.3. *Molecular weight, Western blotting and effects of protease inhibitors*

The purity and minimum molecular mass of the purified diaphorase were assayed by SDS-PAGE. The purified enzyme migrated with an apparent molecular mass of 77 kDa (Fig. 3, lane 2), although several faint bands were also present in the lower molecular mass region. We also examined the effect of protease on purity and molecular mass of the NADPH diaphorase. The diaphorase was purified from the membrane fraction by the same method in the presence of only three antiproteases, PMSF, DIFP and TLCK. The NADPH-dependent NBT reductase activity decreased remarkably during its purification, although NADPH-dependent cytochrome *c* reductase activity was comparatively unchanged. After SDS-PAGE of this NADPH diaphorase, a major protein band migrated with a molecular mass of 68 kDa, and several minor bands were also observed in the region of the higher molecular mass from 77 to 68 kDa (Fig. 3, lane 1). The 68 kDa protein, which seemed to be formed by partial proteolysis of the 77 kDa diaphorase, showed only NADPH-dependent cytochrome *c* reductase activity. These results suggest that proteolysis of the diaphorase during its purification is strongly protected by the addition of protease inhibitor cocktail.

Then, Western blotting analysis was carried out to test whether the native NADPH diaphorase shared antigenic deter-

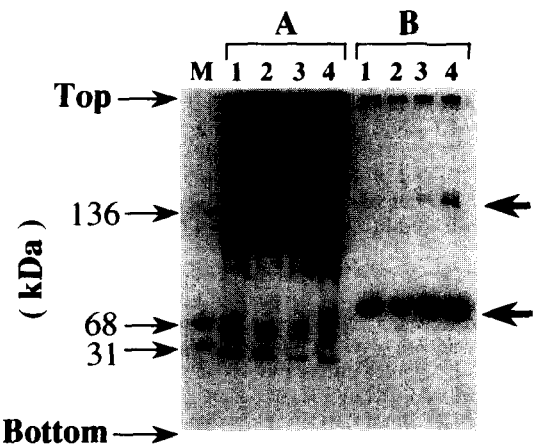


Fig. 2. Activity-staining of NADPH-dependent NBT reductase in the solubilized membrane fraction of undifferentiated and differentiated HL-60 cells. The supernatants (105,000 × *g*, 1 h) of solubilized membrane prepared from undifferentiated and differentiated HL-60 cells were subjected to non-denaturing PAGE (7.5% gel) in the presence of 35 mM OG (50 μg protein/lane). After electrophoresis the gels were used for protein staining (A) with Coomassie brilliant blue, and enzymatic activity staining (B) for NADPH-dependent NBT reductase. The arrows on the right side denote the NBT-stained bands. Gel lanes 1, undifferentiated HL-60 cells without PMA stimulation; 2, undifferentiated HL-60 cells with 0.1 μM PMA stimulation; 3, differentiated HL-60 cells without PMA stimulation; 4, differentiated HL-60 cells with 0.1 μM PMA stimulation. Lane M contained marker proteins (bovine serum albumin dimer, 136 kDa; bovine serum albumin monomer, 68 kDa; carbonic anhydrase, 31 kDa).

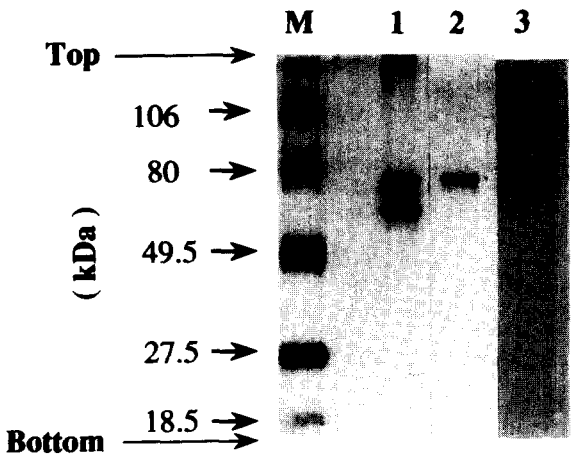


Fig. 3. SDS-PAGE and immunoblot analysis of the purified NADPH diaphorase from the membrane of the differentiated HL-60 cells. The purified NADPH diaphorase was subjected to SDS-PAGE (10% gel), followed by silver staining. Lane 1, purified NADPH diaphorase (0.6 μg/lane) in the presence of PMSF, DIFP and TLCK; 2, purified NADPH diaphorase (0.6 μg/lane) in the presence of PMSF, DIFP, TLCK, and protease inhibitor cocktail. The purified diaphorase (1.5 μg/lane), that was the same sample as lane 2, was electrically blotted to Immobilon PVDF membrane. The PVDF membrane was treated with polyclonal goat IgG anti-microsomal P450 reductase (lane 3). Lane M contained pre-stained marker proteins (phosphorylase B, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa).

minants with the microsomal cytochrome P450 reductase from rabbit liver. The immuno-reacting protein band apparently corresponded with purified NADPH diaphorase with a molecular mass of 77 kDa (Fig. 3, lane 3). The 77 kDa protein seemed to have a specific immunoreactivity against anti-rabbit P450 reductase IgG because of an immediate coloration and a non-reactivity against non-immune IgG. The proteolyzed 68 kDa diaphorase also had an immunoreactivity against anti-rabbit P450 reductase IgG in a manner similar to that reported in previous results [7]. The present results suggest that this NADPH diaphorase seems to have regions that are identical or closely homologous to P450 reductase.

## 4. Discussion

In this paper, we describe the two-stage rapid purification of a membrane-bound NADPH diaphorase in the presence of PMSF, DIFP, TLCK, and protease inhibitor cocktail. The purified membrane-bound diaphorase with its apparent molecular mass of 77 kDa catalyzed NADPH-dependent reduction of both cytochrome *c* and NBT, but it readily underwent partial proteolysis and converted to a 68 kDa enzyme which still held activity for cytochrome *c* reduction but not for NBT reduction. Laporte et al. [3] reported that an NADPH-dependent cytochrome *c* reductase, with a molecular mass of 77 kDa purified from the neutrophil membrane, was also partially cleaved during its purification in the absence of any protease inhibitor, yielding a water-soluble 67 kDa protein with the same substrate specificity and kinetic behavior as the native 77 kDa protein. They could purify the membrane-bound intact 77 kDa reductase in the presence of DIFP alone, though our results (Fig. 3) showed that purification of the diaphorase in the presence of PMSF, DIFP and TLCK yielded a major protein with a molecular mass of 68 kDa. However, the proteolysis of the native diaphorase was effectively blocked by the protease inhibitor cocktail. It remains unclear which protease inhibitor is the most effective. Overall purification and recovery of NADPH-dependent cytochrome *c* reductase were nearly in parallel with those of NADPH-dependent NBT reductase (Table 1). These results suggest that the activities of the NADPH-dependent cytochrome *c* and NBT reductions may be catalyzed by the same diaphorase which is supposed to be induced by the cell differentiation.

Some properties of the purified native enzyme are similar to those of P450 reductase, for example, molecular mass (77 kDa), affinity to 2′,5′-ADP-Sepharose, electron donor specificity for NADPH, specificity for electron acceptors (cytochrome *c*, NBT), prosthetic groups (FAD, FMN), and susceptibility to limited proteolysis yielding a water-soluble 67 kDa protein. Although the subcellular location of the NADPH diaphorase is not clear yet, the enzyme appears to have regions that are identical or closely homologous to P450 reductase in hepatic microsome. Since NO synthase is predominantly found in the cytosol of human neutrophils [16], the 77 kDa protein isolated from the membrane fraction does not appear to be the proteolyzed C-terminal fragment of NO synthase which has an amino acid sequence very close to P450 reductase [17].

Laporte et al. [3] and Miki et al. [4] have already reported that NADPH diaphorase could activate superoxide producing NADPH oxidase activity in a reconstituted assay system. Although the effect of our native 77 kDa protein on superoxide generation was examined in a cell-free assay system, no marked stimulation of the activity was observed. Further experiments are now in progress to study the physiological function of the NADPH diaphorase from the membrane of differentiated HL-60 cells.

Table 1
Purification of NADPH-dependent diaphorase from membrane fraction of DMSO-induced differentiated HL-60 cells

| | Total protein (mg) | Specific activity of (μmol/min/mg protein) | | Purification of (fold) | | Recovery of (%) | |
|---|---|---|---|---|---|---|---|
| | | Cytochrome *c*[a] | NBT[b] | Cytochrome *c*[a] | NBT[b] | Cytochrome *c*[a] | NBT[b] |
| Membrane | 88.05 | 0.056 | 0.083 | 1 | 1 | 100 | 100 |
| Solubilized membrane | 20.45 | 0.204 | 0.288 | 3.64 | 3.47 | 84.64 | 78.81 |
| DEAE-Sepharose CL-6B | 3.85 | 0.865 | 0.935 | 15.45 | 11.26 | 67.53 | 49.25 |
| 2′,5′-ADP-Sepharose 4B | 0.074 | 4.991 | 6.40 | 89.13 | 77.10 | 7.48 | 6.48 |

NADPH-dependent diaphorase activities were measured in the presence of SOD.
[a]NADPH-dependent cytochrome *c* reductase.
[b]NADPH-dependent NBT reductase.

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