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Aqueous two-phase partition and detergent precipitation of a drug-responsive NADH oxidase from the HeLa cell surface

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Abstract

The partitioning behaviour of a drug (capsaicin)-responsive NADH oxidase (tNOX) activity released from HeLa ceIls by low pH treatment followed by heat and proteinase K was determined. When partitioned in a standard 6.4% PEG 3350/6.4% dextran T-500 two-phase system, the bulk of the tNOX activity was in the dextran-rich lower phase. The activity was inhibited by and bound to the triazine dye, Cibacron blue. Affinity partition, where the Cibacron blue was coupled to amino PEG 5000 and added to the first two-phase separation step, resulted in the partitioning of activity to the upper PEG phase. A second partition with PEG-salts resulted in the release of the tNOX from the Cibacron blue–amino PEG enriched phase into the salt-enriched lower phase. The phase-purified protein exhibited anomalous behavior and tended to multimerize in sodium dodecyl sulphate (SDS) prior to SDS-polyacrylamide gel electrophoresis (PAGE). Multimerization appeared to be enhanced by PEG. The multimerization was enhanced with the reduced protein in the presence of detergent prior to SDS–PAGE. In addition, the activity was precipitated by PEG 8000 at concentrations between 6 and 30% by weight. In the presence of or after exposure to PEG 3350 or PEG 8000, the protein could not be detected by Western blot analysis after SDS–PAGE suggesting that the protein failed to enter the gel even though other HeLa cell surface proteins were unaffected. The anomalous multimerization behavior has thus far precluded the use of phase partition as a practical purification step for the oxidase.

Keywords: Aqueous two-phase partitioning; NADH oxidase; Enzymes; Drug-responsive NADH oxidase; Cibacron blue

1. Introduction

A hormone- and growth factor-stimulated NADH oxidase activity of the mammalian plasma membrane that also was resistant to cyanide has been described for rat liver [1,2]. Upon transformation (rat hepatoma), the growth factor and hormone-responsiveness of the plasma membrane NADH oxidase activity was absent or greatly reduced [3,4]. Instead, the activity was now responsive to antitumour drugs such as adriamycin [5].

Similarly, a drug-responsive isoform of NADH oxidase (designated tNOX) is resident to the cell surface of HeLa cells [6,7], a human transformed cell line of cervical carcinoma derivation. The cancer or tumour form of the activity also catalyses membrane protein disulphide–thiol interchange [8] with the concomitant reduction of protein disulphides (or under certain circumstances, reduction of molecular oxygen). tNOX (tumour-associated NOX) activity is defined as that portion of the total NOX activity

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inhibited by capsaicin (8-ethyl-*N′*-vanillyl-6-nonemide) [7] or by the antitumour sulphonylurea, *N*-(4-methylphenylsulphonyl) - *N′* - (4-chlorophenyl) urea (LY 181984) [6]. Normally, maximum inhibition of tNOX activity is given by 0.1 μ*M* of capsaicin [7].

Because tNOX is a cancer-specific activity of potential importance to cancer diagnosis [9,10] and therapy [5], a purification scheme for processing large quantities of the activity from HeLa S cells grown in culture would be of considerable utility. The tNOX is purified as a 33 to 34 kDa protein from a fraction released from intact HeLa S cells by treatment with low pH (sodium acetate, pH 5) [11].

2. Experimental

2.1. Reagents and chemicals

Poly(ethylene glycol) (PEG) 3350 and 8000 were from Fisher Scientific (Pittsburgh, PA, USA). Dextran T-500 was from Pharmacia Biotech, Inc. (Alameda, CA, USA). Aminated PEG 5000 was from Shearwater Polymers (Huntsville, AL, USA). The other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of HeLa cells and cell-free extracts

HeLa S cells, grown commercially (Cellex Biosciences, Minneapolis, MN, USA), were collected by centrifugation and shipped frozen in 0.1 *M* sodium acetate, pH 5, in a ratio of 1-ml packed cell volume to 1-ml of sodium acetate. The cells were thawed at room temperature, resuspended and incubated at 37°C for 1 h to release the protein. The cells were removed by centrifugation at 31 700 *g* for 60 min (Sorvall) and the cell-free supernatants were refrozen and stored in 1-ml aliquots at −70°C.

For heat treatment, 1-ml aliquots of the above supernatant material were thawed at room temperature and heated to 50°C for 10 min. The denatured proteins were removed by centrifugation (4 640 *g*, 5 min, Eppendorf centrifuge in 1-ml aliquots). Full activity was retained from this step [11].

For protease treatment, the pH of the heat-stable supernatant was adjusted to 7.8 by addition of 0.1 *M* sodium hydroxide. *Tritirachium album* proteinase K was added (4 μg ml^{-1}) and incubated at 37°C for 1 h with full retention of enzymatic activity and drug response [12]. The reaction was stopped either by freezing for determination of enzymatic activity or by addition of 0.1 *M* phenylmethylsulphonyl fluoride (PMSF) in ethanol to yield a final concentration of 10 m*M* PMSF. Activity was measured by oxidation of NADH (decrease in absorbance at 340 nm). The inhibition of NADH oxidation by capsaicin was taken as a specific measure of tNOX activity.

2.3. Cibacron blue binding

To determine if Cibacron blue binding influenced the NADH oxidase activity of the pH 5-released supernatant fraction from HeLa cells after heat and proteinase K treatment, 10^{-10} to 10^{-5} *M* of Cibacron blue was added and the samples were assayed for NADH oxidase activity.

2.4. Affinity chromatography

As a means of determining if the NOX activity of the pH 5-released supernatant fraction after heat and proteinase K treatment bound to the Cibacron blue, a Blue Sepharose CL6B High Flow Column (1 ml) (Pharmacia Biotech, Alameda, CA, USA) was used to fractionate the low pH supernatant from the HeLa cells after heat and proteinase K treatment. The column was equilibrated with 20 m*M* Tris-HCl at a flow-rate of 12 ml h^{-1}. The sample (0.5 ml) was applied to the column. The void volume was discarded and the absorbed protein was eluted with 0.5 *M* NaCl after a previous wash with 0.1 *M* NaCl. The wash and one-half ml fractions of the 0.5 *M* NaCl eluate were collected and assayed for capsaicin-inhibited NADH oxidase activity.

2.5. Two-phase partitioning

Partition studies are described for a 10 g system at 4°C. Stock solutions of the components were placed into 15 ml glass (Corex) tubes together with HeLa cell pH 5-released supernatant fraction after heat and proteinase K treatment. The contents of the phase systems were mixed on a Vortex agitator, stirred for

20 min and then centrifuged for 5 min in a swing-out rotor (Sorvall HB-4) at 750 *g* to speed phase formation. The phases were separated and the volume of each phase was estimated. Samples were removed from the top and bottom phases for determination of enzymatic activity and protein content and for analysis by SDS-PAGE. The partition coefficient, *K*, was defined as the ratio of the enzymatic activity in the top phase to that in the bottom phase.

2.6. Affinity phase partition with Cibacron blue amino PEG 5000

Cibacron blue coupled to amino PEG 5000 (100 mg) was added to the standard PEG two-phase aqueous system. The resulting top phase was mixed with a solution of PEG 8000 and salts resulting in dissociation of the Cibacron blue–amino PEG 5000–enzyme complex with Cibacron blue–amino PEG remaining in the top phase and the capsaicin-inhibited NOX activity being partitioned to the lower phase.

2.6.1. Cibacron blue amino-PEG 5000

The coupling of the aminated PEG 5000 to Cibacron blue was as described by Bückman et al., [13]. In a nearly equimolar ratio, 10 g of aminated PEG 5000 and 2.5 g of pure Cibacron Blue F3G-A were dissolved in 100 ml water. The solution was stirred at 60°C while maintaining the pH at 11 with 1 *M* potassium hydroxide. After 16 h, the mixture was neutralized with 2.5 *M* HCl. The free, unreacted dye was removed by gel permeation. The gel permeation column (5×52 cm) packed with Sephadex G-50 gel (fine) was loaded with 50 ml of reaction solution, diluted to a concentration of 5% (w/w) PEG, and eluted with 0.1 *M* NaCl solution at a flow-rate of 100 ml h^{-1}. The unreacted aminated PEG and free Cibacron blue F3G-A passed through the column more slowly than the derivative owing to their lower molecular masses. The sodium chloride in the eluate was removed by dialysis against distilled water. The solution was concentrated by evaporation under vacuum at 60°C and stored at 4°C. The Cibacron blue F3G-A content of the product was estimated from the absorbance of the water solution at 612 nm (molar absorptivity of 13 000 M^{-1} cm^{-1}).

2.6.2. First aqueous phase extraction

The phase components were stored at 4°C and added at that temperature. Phase system 1 was constituted as follows for a 10-g system in a 15-ml Corex centrifuge tube: 3.2 g 20% dextran T-500, 1.6 g 40% PEG 3350, 0.2 ml 0.2 *M* potassium phosphate buffer, pH 7.2, cold distilled water to 8.75 g and sample to 10 g [14]. Cibacron blue–amino PEG 5000 (100 mg) was added where indicated. The contents of the two-phase system were mixed by Vortex mixing at room temperature for 20 min and then centrifuged at 750*g* for 5 min using a Sorvall HB-4 rotor. The PEG phase (top phase) was removed and used in the second extraction step.

2.6.3. Second aqueous phase extraction

An equal volume of a solution of PEG 8000 (300 mg ml^{-1}) and salts (NaCl, 40 mg ml^{-1}; NaH_2PO_4, 80 mg ml^{-1}; and K_2HPO_4, 40 mg ml^{-1}) was added to the top phase. The mixture was stirred for 20 min and then centrifuged at 1000 *g* for 5 min (Sorvall HB-4 rotor). The salt-enriched phase (bottom phase) was obtained by removal of the PEG phase. The re-extraction of the enzyme into the salt-rich bottom phase permitted the removal of the Cibacron blue–amino PEG which was retained in the upper phase. The bottom phase from the second extraction was desalted by several concentration steps with an ultrafiltration module (Centricon 10) and subsequent dilution with 10 m*M* potassium phosphate, pH 7.0.

2.7. Spectrophotometric assay

NADH oxidase activity was determined at 37°C as the oxidation of NADH measured at 340 nm with 430 nm as reference using an SLM Aminco DW-2000 spectrophotometer in the dual wavelength mode of operation or a Hitachi U3210 spectrophotometer at 340 nm with stirring and continuous recording over 5-min intervals once steady-state rates were obtained. The reaction mixture contained in a final volume of 2.5 ml consisted of 50 m*M* Tris–Mes buffer (pH 7.2), 1 m*M* KCN to inhibit any potential mitochondrial oxidase activity and 150 μ*M* NADH. A millimolar extinction coefficient of 6.22 was used to calculate the rate of NADH disappearance.

Proteins were determined by the bichinchoninic

acid (BCA)/copper assay [15] using bovine serum albumin as standard.

2.8. Analytical SDS–PAGE electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was with the buffer system of Laemmli [16] on acrylamide slab gels. Proteins were denatured in sample buffer by boiling for 3 min and analyzed by SDS–PAGE (10% acrylamide). The gels were stained for protein using silver [17].

2.9. Immunoblotting

Proteins were separated on 10% SDS–PAGE and then transferred by electroblotting onto nitrocellulose. To block unspecific antibody binding sites, the blot was placed in a solution of 5% bovine serum albumin, 10 m*M* Tris–HCl, 150 m*M* NaCl and 0.1% Tween 20 (TBS-T) for 15 min. The blocking solution and the blot were placed in the primary antibody solution (1:1000) overnight at 4°C with shaking. The blots were washed with TBS-T four times for 15 min each after which the blots were placed into secondary antibody (goat anti-rabbit linked to alkaline phosphatase, Jackson ImmunoResearch Laboratories, W. Grove, PA, 1:25:000 in TBS-T) for 30 min at room temperature with shaking. The blot was washed with TBS-T three times for 15 min each and placed in a mixture of 0.33 mg ml^{-1} nitro blue tetrazolium and 0.16 mg ml^{-1} of 5-bromo-1-chloro-3-indolyl phosphate prepared in 100 m*M* Tris, pH 9.5, containing 100 m*M* NaCl and 5 m*M* $MgCl_2$ and incubated with shaking until the purple colour of positive bands appeared. The colour development reaction was stopped by placing the blots in 10 m*M* Tris, pH 8, containing 5 m*M* EDTA.

3. Results

The NADH oxidase (NOX) activity at the external surface of HeLa cells that was released by treatment at low pH exists in two forms. One, the cancer form, is inhibited by certain anticancer drugs such as capsaicin. The other, the non-cancer form, is resistant to inhibition by capsaicin. When HeLa cells were treated with 0.1 *M* sodium acetate, pH 5, the capsaicin-inhibited activity was released [11]. After treatment with 0.1 *M* sodium acetate, pH 5, the capsaicin-inhibited NADH oxidase activity remaining at the cell surface was reduced or absent and the bulk of the capsaicin-inhibited activity was present in the cell-free supernatant.

The capsaicin-inhibited NOX activity (tNOX) of HeLa cells was resistant to proteases and to heating [11,12]. Therefore, as an initial purification step, the cell-free extracts were heated for 10 min at 50°C and centrifuged to remove denatured proteins followed by incubation for 1 h with 4 μg ml^{-1} proteinase K. After this step, there was approximately a 14-fold purification of the protein with no loss of capsaicin-inhibited activity [11].

To attempt further purification of the tNOX protein we used aqueous two-phase partition. Aqueous two-phase partition with a 6.4% system of PEG 3350/Dextran T-500 resulted in an unequal distribution of both NOX activity and protein between the upper and lower phases (Table 1). The bulk of the NOX activity partitioned into the lower phase

Table 1
Aqueous two phase partition of a capsaicin-inhibited NADH oxidase (NOX) released from HeLa cells by low pH treatment followed by heat and proteinase K with a 6.4% PEG-Dextran system comparing PEG 3350 and PEG 8000

	Starting fraction	PEG 3350/Dextran		PEG 8000/Dextran	
		Upper	Lower	Upper	Lower
Total activity (nmol min^{-1} ml^{-1})	19.2±1.7	2.0±0.2	19.5±1.5	3.1±0.9	17.0±2.0
Capsaicin-inhibited activity (nmol min^{-1} ml^{-1})	9.6±0.9	0.5±0.2	9.9±0.9	0.9±0.3	8.7±3.3
Protein (mg ml^{-1})	3.5±0.3	0.6±0.2	2.2±0.4	0.7±0.15	2.1±0.4

Results are from three experiments±standard deviations.

and this activity was inhibited about 50% by capsaicin. The overall enrichment of the capsaicin-inhibited activity in the lower phase was approximately 1.7-fold. Similar distributions of total and capsaicin-inhibited NOX activities as well as protein were obtained with PEG 8000 compared to PEG 3350 (Table 1). The partition coefficients (K) for total and capsaicin-inhibited activities were 0.10 and 0.05, respectively, for PEG 3350 and 0.18 and 0.1, respectively, for PEG 8000.

To increase the efficiency of the two-phase separation, Cibacron blue conjugated to amino PEG 5000 was added to the phase system. Cibacron blue inhibited NOX activity (Fig. 1). NADH oxidase activity was inhibited in proportion to the logarithm of Cibacron blue concentration with about 90% inhibition of activity at 10^{-5} M (Fig. 1). When the activity released from HeLa cells by low pH treatment followed by heat and proteinase K treatment was fractionated by affinity chromatography using a column of Cibacron blue linked to Sepharose, the activity bound. After two washes with 0.1 M NaCl, the activity was eluted with 0.5 M NaCl. Fraction 4 had the greatest activity and was inhibited by more than 60% by 1 μM capsaicin and approximately 75% by 100 μM capsaicin (Fig. 2).

When Cibacron blue coupled to amino PEG 5000 was added to the PEG 3350/Dextran T-500 aqueous two-phase system, the capsaicin-inhibited tNOX activity partitioned quantitatively into the PEG-rich upper phase (Table 2B) in contrast to partition in the absence of Cibacron blue–amino PEG where most of the activity remained in the lower phase (Table 2A). In a second two-phase separation of the first upper phase using a PEG-salt system, NADH oxidase partitioned quantitatively into the salt-rich lower phase (Table 2B). The salt dissociated the tNOX from the Cibacron blue–amino PEG 5000 and the latter remained in the upper phase of the salt-PEG system. This step resulted in a three- to five-fold purification of the capsaicin-inhibited NADH oxidase activity.

When analyzed by SDS–PAGE, the fractions

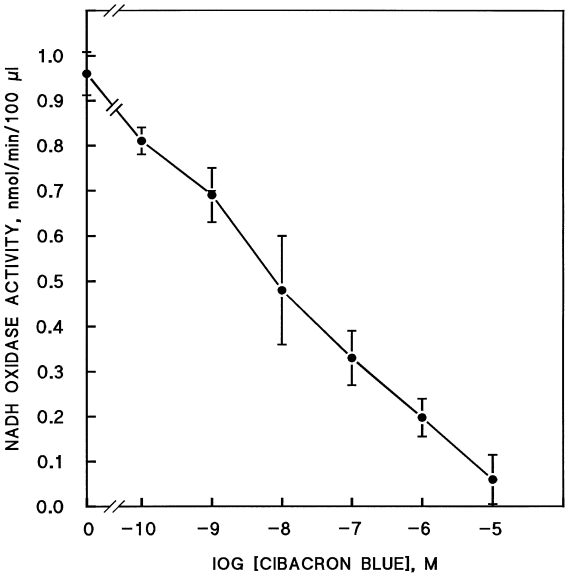

Fig. 1. Dose response to the logarithm of Cibacron blue concentration of capsaicin-inhibited NADH oxidase activity released from HeLa cells by low pH treatment followed by heat and proteinase K. Activity was about 90% inhibited at a Cibacron blue concentration of 10^{-5} M.

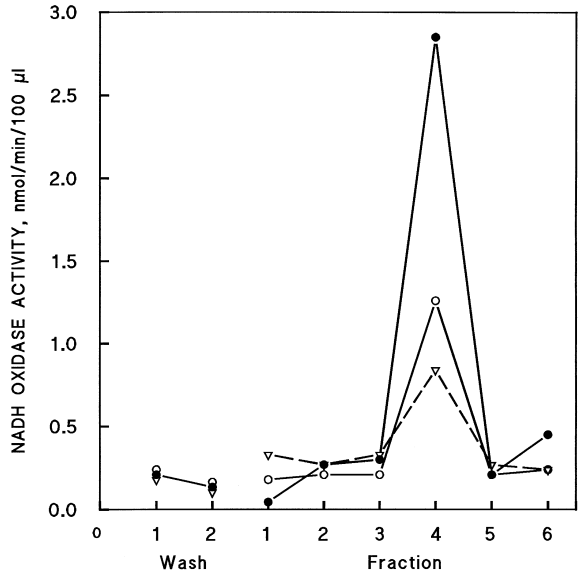

Fig. 2. NADH oxidase activity of fractions of a capsaicin-inhibited NADH oxidase activity released from HeLa cells by low pH treatment followed by heat and proteinase K bound to a Cibacron blue Sepharose CL6B column and eluted with 0.5 M sodium chloride following a wash with 0.1 M sodium chloride. NADH oxidase activities were determined for 50-µl portions of the column eluate in the absence (solid circles and solid line) or presence of 1 μM (open circles and solid line) or 100 μM (open triangles and dotted line) capsaicin.

Table 2
Total and capsaicin-inhibited NADH oxidase activity released from HeLa cells by low pH treatment followed by heat and proteinase K separated by aqueous two-phase partition with and without Cibacron blue–PEG 5000

	Total activity (nmol min^{-1} ml^{-1})	Inhibited by 1 μM capsaicin	Protein (mg ml^{-1})
Starting material	20.5	11.2	3.2
A. No Cibracron blue–PEG 5000			
First partition			
U_1	7.0	2.6	1.2
L_1	11.1	5.1	1.5
Second partition of upper phase			
U_2	1.6	0	0.8
L_2	26.2	2.9	0.4
B. Plus Cibacron blue–PEG 5000			
First partition			
U_1	7.1	5.6	1.0
L_1	3.8	0	1.3
Second partition of upper phase			
U_2	3.6	0	0.5
L_2	21.8	8.0	0.3

The second partition was by salt partition.

obtained with the Cibacron blue–amino PEG 5000 fractionation showed a pattern of bands from the dextran-rich lower phase on silver-stained gels (Fig. 3, lane 3) nearly identical to that of the starting fraction (Fig. 3, lane 1). With the first upper phase (Fig. 3, lane 2) and the second lower phase (Fig. 3, lane 4), a simplified pattern of 12 to 14 major bands was observed after dissociation of the NOX from the Cibacron blue–amino PEG.

To identify the tNOX protein, we used monospecific rabbit antisera raised to the tNOX protein purified from culture media conditioned by growth of HeLa cells [18]. Western blot analyses of the SDS–PAGE in parallel to those of Fig. 3 revealed immunoreactive bands at 33–34 kDa (putative tNOX monomer) and 29 kDa (a proteolytic core fragment of the 34 kDa band) and a doublet at 66/68 kDa which may represent dimers of the 33 kDa and 34 kDa bands (Fig. 4, lane 1). The upper phase (Fig. 4, lane 2) which contained the bulk of the capsaicin-inhibited tNOX activity revealed immunoreactive bands at 53/54 kDa (arrow) representing heterodimers of the 33–34 kDa monomers plus a ca. 20 kDa glycine-rich protein (See Fig. 5). The material retained in the lower phase (Fig. 4, lane 3) in addition to the putative 66 and 68 kDa dimer bands revealed bands of uncertain origin at about 49 and 58 kDa not present in the starting material but presumably representing some form of a tNOX multimer with other proteins. After the second partition and dissociation from the Cibacron blue–amino PEG 5000 (Fig. 4, lane 4), the only immunoreactive material remaining was a minor band at 68 kDa representing the 34 kDa dimer.

Based on the present study and considerable additional work, the pattern of dimers, tetramers and multimers that characterizes the tNOX protein is summarized in Fig. 5. The apparent molecular mass of the processed monomer was estimated from SDS–PAGE to be 33–34 kDa. The protein forms both homo- and heteromultimers stable to SDS–PAGE. Homomultimers include dimers of 66/68 kDa and tetramers of 132/136 kDa. A proteolytic core fragment (29 kDa) forms a 58 kDa dimer and a tetramer of 116 kDa. The 33 kDa and 29 kDa proteins also form heteromultimers with a 20 kDa glycine-rich protein (unpublished) resulting in heterodimers of either 49 kDa (29 kDa+20 kDa) or 53 kDa (33 kDa+20 kDa). These heterodimers are the principal forms found after hydrophobic interaction chromatography and may be dissociated into their respective monomers when the bands are excised and subjected to a second PAGE without heating (F. Yantiri, Purdue University, results unpublished).

The identities of each of the immunoreactive multimer forms of Fig. 5 has been established from dissociation into monomers upon re-electrophoresis, formation from purified monomer added in solution and from amino acid composition. The amino acid compositions of the 33 kDa monomer and that of the 29 kDa core fragment are similar while the amino acid composition of each of the proposed monomer and dimerized forms are indistinguishable.

The loss of the immunoreactive tNOX bands was unaffected by added dextran T-500 (Fig. 6A, lane 2) but enhanced by added PEG 8000 (Fig. 6A, lane 3) compared to starting material (Fig. 6A, lane 1). In this experiment, the 33–34 kDa monomers were missing even from the starting material and the immunoreactive components were present as dimers and tetramers. The proteolytic core fragment at 29

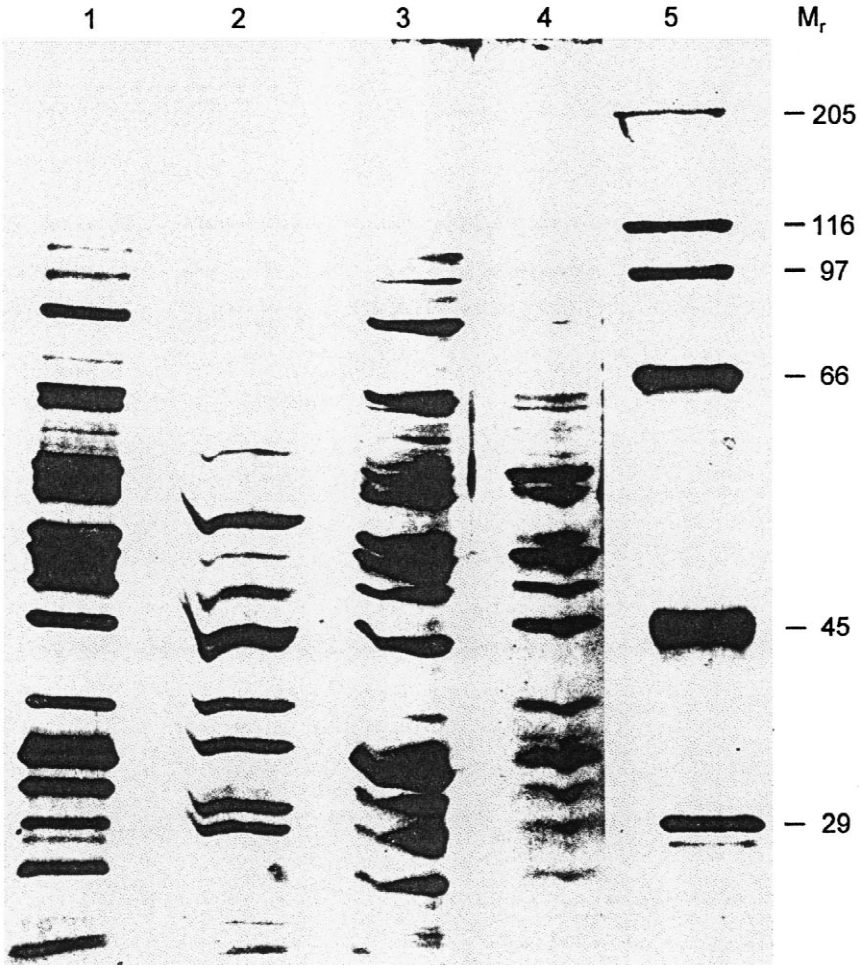

Fig. 3. Silver-stained 10% polyacrylamide gel of the fractions released from HeLa cells by low pH treatment followed by heat and proteinase K following separation by an aqueous two-phase system containing Cibacron blue conjugated to amino PEG 5000. Lane 1, starting material). Lane 2, first upper phase. Lane 3, first lower phase. Lane 4, second lower phase. Lane 5, molecular mass standards, carbonic anhydrase (29 kDa), chicken egg ovalabumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase b (97 kDa); *E. coli* β-galactosidase (116 kDa) and rabbit muscle myosin (205 kDa). The protein content of the second upper phase was negligible. Molecular mass is indicated in kDa.

kDa did remain, however. The loss of immunoreactive material was observed despite a normal pattern of protein bands after SDS–PAGE and silver staining (Fig. 7A, lanes 1–3).

The tNOX activity was affected neither by salt (not shown) nor by PEG 8000 (Table 3) when added to the starting material released from cells by low pH treatment and after heat and proteinase K treatment (Table 3). There was a tendency for the tNOX protein to precipitate in the presence of PEG 8000. Results of a fractional precipitation with PEG 8000 are shown in Table 4. Most of the activity remained in solution with 6% PEG 8000 but approximately 60% of the activity was precipitated by 30% PEG 8000 with 40% remaining in the supernatant. There was approximately 85% recovery of total activity (sum of the total activities in the pellets plus that of the 30% PEG 8000 supernatant/total activity of the precleared starting material) by the fractional precipitation with PEG 8000. The majority of the

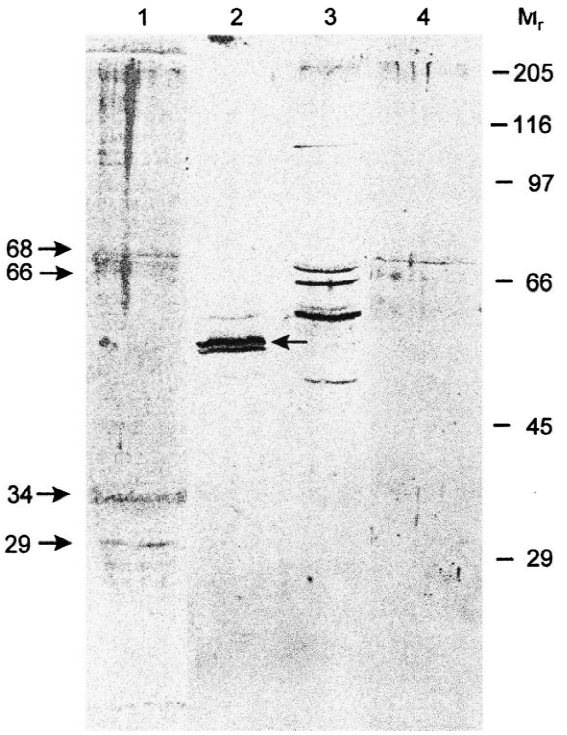

Fig. 4. Western blot analyses of a 10% PAGE of the fractions of Fig. 3 released from HeLa cells by low pH treatment followed by heat and proteinase K following separation by an aqueous two-phase system containing Cibacron blue conjugated to amino PEG 5000. Monospecific rabbit antisera raised to the NOX protein [13] was used for immunostaining. Lane 1, starting material. Lane 2, first upper phase. Lane 3, first lower phase. Lane 4, second lower phase. The major two bands in lane 2 are at 53/54 kDa (arrow). Molecular mass is indicated in kDa.

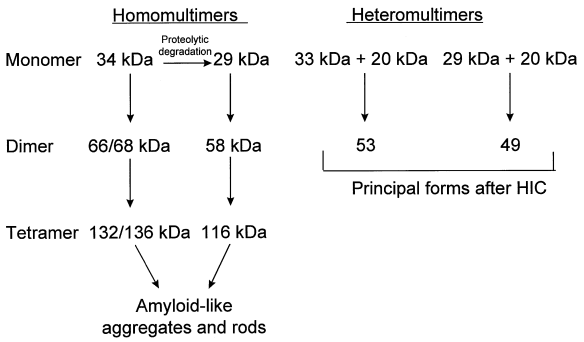

Fig. 5. Pattern of dimers and tetramers derived from the 33 and 34 kDa monomers with capsaicin-inhibited NADH oxidase activity released from HeLa cells by low pH treatment. The apparent molecular mass of the processed monomer has been estimated from SDS–PAGE to be 33–34 kDa. Based on the use of monospecific antisera [18] and analyses by re-electrophoresis, the protein has been shown to form both homo- and heteromultimers stable to SDS–PAGE. The predominant homomultimers are dimers of 66/68 kDa and tetramers of 132/136 kDa. A proteolytic core fragment (29 kDa) has also been identified. It forms a dimer of 58 kDa and a tetramer of 116 kDa. The 33 kDa and 29 kDa proteins form heteromultimers with a ca. 20 kDa glycine-rich protein (unpublished) resulting in heterodimers of either 49 kDa (29 kDa+20 kDa) or 53 kDa (33 kDa+20 kDa). These heterodimers are the principal forms found after hydrophobic interaction chromatography and may be dissociated into the monomers indicated above when the bands are excised and subjected to a second SDS–PAGE without heating (F. Yantiri, Purdue University, results unpublished).

capsaicin-inhibited activity was in the 30% PEG 8000 pellet (Table 53).

Western blot analysis of the PEG 8000 pellets and supernatants revealed no immunoreactive bands (Fig. 6B, lanes 5 and 6). In the starting material, the immunoreactive protein was present almost exclusively as the 34 kDa monomer (Fig. 6B, lane 4, arrow).

Silver staining of SDS–PAGE run in parallel to that of Fig. 6 revealed an abundance of protein bands in the pellet after precipitation with 30% PEG (Fig. 7B) demonstrating that the loss of the immunoreactive tNOX protein was selective.

4. Discussion

The technique of aqueous two-phase partition has been used extensively to purify proteins from cell supernatant solutions [19]. This method which involves mixing the proteins to be separated with two polymers, such as polyethylene glycol and dextran, that form phase-unstable mixtures has been useful for the large-scale purification of several different dehydrogenases and kinases [20–25]. In this report, we have attempted to adapt this technique to the purification of a cancer isoform of NADH oxidase (tNOX) from the cell surface of HeLa cells. Affinity partitioning was used where the triazine dye, Cibacron blue, was coupled to amino PEG 5000 and added to the first partitioning step. The top phase was removed and added to an equal volume of a

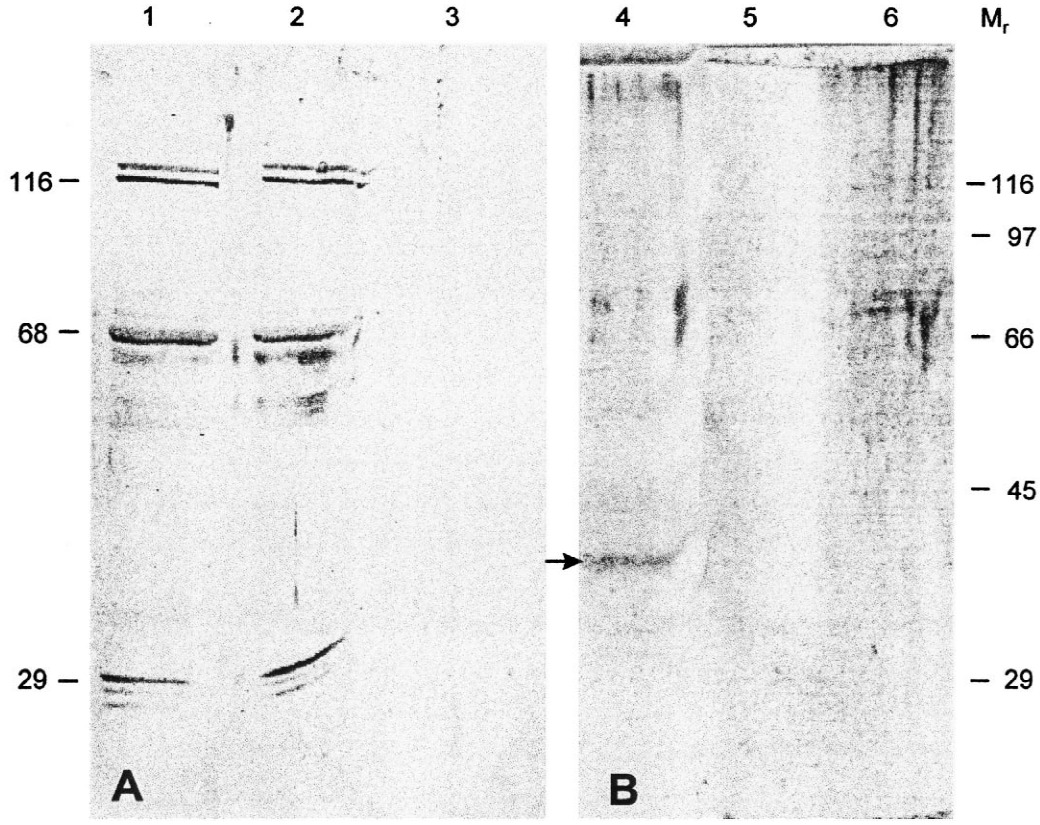

Fig. 6. Western blots with monospecific rabbit antisera [13] illustrating the effect of PEG 8000 on electrophoretic mobility of immunoreactive tNOX forms. (A) Starting fraction released from HeLa cells by low pH treatment followed by heat and proteinase K resolved on 10% SDS–PAGE in the presence or absence of an equal volume (20 μl) of either 20% dextran T-500 or 20% PEG 8000 prior to mixing with the SDS-containing sample buffer. Lane 1, starting material. Lane 2, added dextran T-500. Lanes 3, added PEG 8000. (B) Comparison of starting fraction with the 30% PEG pellet and the 30% PEG supernatant. Lane 4, starting fraction containing the capsaicin-inhibited NADH oxidase released from HeLa cells by low pH treatment following heat and proteinase K treatment compared to the 30% PEG 8000 precipitate of Table 5 (lane 5) and the 30% PEG supernatant of Table 5 (lane 6). The immunoreactive protein was present almost exclusively as the 34 kDa monomer (arrow). Molecular mass is indicated in kDa.

solution of PEG 8000 and salts for a second phase extraction which resulted in the release from the Cibacron blue–amino PEG 5000 and repartition into the lower phase.

After phase partitioning, we encountered an anomalous behaviour of the tNOX protein. The anomalous behaviour appeared somehow to be related to an interaction of the protein with the PEG that resulted in a failure of the partially purified protein to resolve on SDS–PAGE and its eventual disappearance from solution. While added PEG did not block enzymatic activity, it appears to have enhanced multimerization of the protein especially when the protein was reduced and dissolved in SDS prior to gel electrophoresis. This argument is supported by the ability of PEG to preferentially precipitate the capsaicin-inhibited activity from the starting material and that the precipitation was enhanced by the addition of 100 μ*M* glutathione (not shown).

A precedent for decreased protein solubility in the presence of PEG 8000 is found in the prion literature where precipitation by PEG 8000 was used to purify

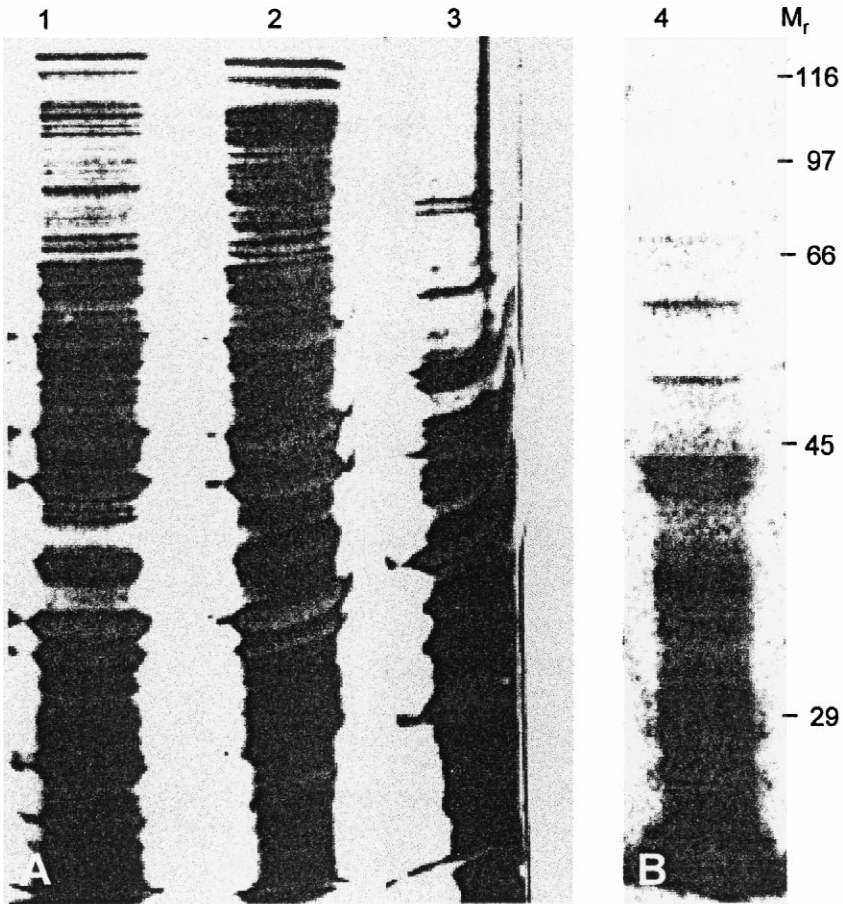

Fig. 7. Silver stained SDS–PAGE corresponding to the Western blot of Fig. 6A. Starting fraction released from HeLa cells by low pH treatment followed by heat and proteinase K resolved on 10% SDS–PAGE in the presence or absence of an equal volume (20 μl) of either 20% dextran T-500 or 20% PEG 8000 prior to mixing with the SDS-containing sample buffer. Lane 1, starting material. Lane 2, added dextran T-500. Lane 3, added PEG 8000. B. Lane 4, 30% PEG 8000 precipitate. Despite the presence of >14 major protein bands, no immunoreactive material was present (cf., Fig. 6B, lane 6). The SDS–PAGE of the starting material and of the 30% PEG 8000 supernatant were indistinquishable from those of lanes 1 and 2. Molecular mass is indicated in kDa.

Table 3
Effect of preincubation with PEG 8000 on NADH oxidase activity inhibited by capsaicin of fraction released from HeLa cells by low pH treatment followed by heat and proteinase K

	NADH oxidase activity (nmol min^{-1} mg^{-1} protein)	
	No addition	+PEG 8000
No addition	1.0±0.3	1.4±0.4
1 μ*M* capsaicin	0.6±0.1	1.1±0.2
100 μ*M* capsaicin	0.4±0.2	0.9±0.2

A reagent blank was subtracted. Results are from three determinations±standard deviations.

the infective units [26,27]. The phenomenon was encountered during the large scale purification of prions [27]. If EDTA and dithiothrietol were added at a concentration of 1 m*M* and Triton X-100 and sodium deoxycholate (DOC) were added at ratios of 4:1 and 2:1 on a detergent:protein (w/w) basis, the detergent extraction could be precipitated with 8% PEG 8000 [26].

Prusiner et al. [26] reported that precipitation of the scrapie agent by PEG from suspensions containing a variety of detergents. The scrapie agent was readily precipitated with PEG from suspensions

Table 4
Fractional precipitation with PEG 8000 of NADH oxidase activity released from HeLa cells by low pH followed by heat and proteinase K

	Total activity (nmol min^{-1} ml^{-1})	Cumulative percent insoluble
HeLa cells	34.6	
Precleared	41.6	
0–6% PEG 8000 pellet	2.2	5
0–6% PEG 8000 supernatant	34.6	
6–12% PEG 8000 pellet	5.1	18
6–12% PEG 8000 supernatant	26.2	
12–18% PEG 8000 pellet	7.7	36
12–18% PEG supernatant	20.5	
18–30% PEG 8000 pellet	10.9	60
18–30% PEG 8000 supernatant	9.6	

containing octyl glucoside, Brij 35, sarkosyl or DOC. Precipitations from suspensions containing Triton X-100 or sulphobetaine 3-14 were not observed. However, addition of DOC or sodium dodecylsulphate (NaDod SO_4) to Triton X-100 suspensions did result in precipitation of the scrapie agent [26].

Electron microscopy of fractions from the Triton X-100/NaDodSO_4-precipitated material revealed the presence of aggregates consisting of amorphous material and flattened rod-like structures [27]. The behaviour of PrP^{sc} and PrP 27-30 are similar to other amyloidogenic proteins which require partial proteolysis before amyloid formation is observed [28–30]. In parallel with the prion results, the loss of the tNOX protein from solution probably takes place in a similar manner.

The ultrastructural appearance of the prion rods not only is similar to that of amyloid [27] but is similar in appearance to rods formed during the precipitation of the tNOX protein. The NOX protein when purified does readily polymerize into 10-nm wide amyloid rods [11] resembling those of the scrapie prion formed under similar conditions [27]. It is possible that a similar type of polymerization takes place in the SDS prior to PAGE to account for the apparent loss of activity. The prion protein becomes insoluble in SDS and this insolubility is enhanced by PEG 3350 or PEG 8000 and heat. Our protein has been both heated and treated with PEG 3350 or PEG 8000. As with tNOX reported here, apparent dimers and higher molecular mass multimers of PrP^{sc} were evident on the immunoblots after PEG 8000 precipitation [31]. PrP 27-30 polymerized into rod-shaped structures as well in the presence of detergent [27].

PEG precipitation has been widely employed for purification of both proteins and viruses [32,33]. Precipitation of proteins by PEG has been reported to be most selective at 10–20°C with the pH between 3 and 5 [32]. PEG also has been employed to precipitate membrane proteins such as Ca^{2+}-ATPase from detergent mixtures [34].

We were unable to use either the PEG precipitation procedure or affinity partition with Cibacron blue–amino PEG 5000 to advantage in the present study because of the strong tendency of the precipitated tNOX protein to form multimers. In the mean-

Table 5
PEG 8000 precipitation of NADH oxidase activity inhibited by capsaicin released from HeLa cells by low pH treatment followed by heat and proteinase K

	NADH oxidase activity, nmol/min/ml	
	Total	Inhibited by 1 μM capsaicin
Starting material	25.0	10.6
30% PEG 8000 pellet	12.8	6.4
30% PEG 8000 supernatant	8.32	2.6

time, we have gone ahead with the purification of the tNOX protein using hydrophobic interaction chromatography as the final step and ion-exchange chromatography to replace the aqueous two-phase partitioning with Cibacron blue–amino PEG. A problem, similar to that encountered with the protein purified by affinity phase partition, again was encountered with the ion-exchange/hydrophobic interaction-purified-proteins. The latter tended to multimerize in the SDS and often either did not enter the gel or resolved as homo- and heteromultimers of higher molecular masses (F. Yantiri, results unpublished). Heating often appeared to enhance multimerization as well. Until the problem of multimerization in the SDS is resolved, final purification and characterization of the NOX protein and perhaps expression of the NOX protein in bacteria will remain a problem.

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