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Papain proteolysis releases a soluble NADPH dependent diaphorase activity from bovine neutrophil membranes

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Abstract An NADPH dependent cytochrome *c* reductase has been purified from resting bovine neutrophil membranes. A high degree of purification, approaching homogeneity, is indicated by the presence of a single 75 kDa protein band on silver stained SDS-PAGE (10%). The purified protein catalyzes as well an NADPH dependent reduction of iodonitrotetrazolium violet (INT). Limited papain digestion of the purified preparation produces a 65 kDa product which retains both enzymatic activities. In a similar fashion papain digestion of the plasma membrane bound protein generates a fully active *soluble* NADPH dependent INT and cytochrome *c* reductase preparation (65 kDa). Proteolytic cleavage would appear to occur at a protein-membrane anchor remote from the proteins catalytic site. The cytochrome *c* reductase acts independently of the O_2^- generating cytochrome b_{558}, a leukocyte plasma membrane protein which also catalyzes an NADPH dependent INT reduction.

Key words: NADPH dependent diaphorase; Papain proteolysis; Iodonitrotetrazolium violet; Flavocytochrome b_{558}; Neutrophil

1. Introduction

The membrane located NADPH oxidase complex of phagocytes catalyzes a one electron reduction of oxygen, producing superoxide anion (O_2^-), the primary source of a series of microbicidal oxidants (for reviews see [1–3]). The oxidase is dormant in resting neutrophil with the complete activation process yet to be clarified. It is now generally believed that the single membrane component of the oxidase complex, flavocytochrome b_{558}, contains all the redox centers required for superoxide production [4–9]. Several groups have reported that an additional flavin containing dehydrogenase from rabbit peritoneal neutrophils and pig neutrophils is able to enhance superoxide production in a cell-free system [10–12]. Nisimoto et al. [13,14] have purified an NADPH-cytochrome *c* reductase from human neutrophils and HL-60 cells but did not observe a marked stimulation of superoxide generation by the purified reductase.

A previous paper from our laboratory demonstrated that there are two distinguished diaphorase activities (NADPH dependent iodonitrotetrazolium violet (INT) reductases) existing in the plasma membranes of bovine neutrophils [15]. One of these is associated with the flavocytochrome b_{558} and likely represents a partial reaction of the electron transport chain of the NADPH oxidase [16]. The other INT reductase activity cofractionated with an SOD insensitive NADPH-cytochrome *c* reductase. The latter INT reductase does not share the cofactor requirements of the NADPH oxidase nor is it inhibited by the potent oxidase inhibitor phenylarsine oxide [15–17]. We have now purified the latter INT reductase protein essentially to homogeneity and confirm that it is as well a cytochrome *c* reductase. Preliminary experiments using neutrophil plasma membranes revealed that a 'solubilized' cytochrome *c* reductase was liberated from the membranes by partial digestion with papain. This 'solubilized' cytochrome *c* reductase was able to catalyze as well an NADPH dependent INT reductase activity. It was however uncertain whether the 'solubilized' cytochrome *c* reductase originated from a cleavage of cytochrome b_{558}, which in its modified form might act as a cytochrome *c* reductase or directly from the membrane bound cytochrome *c* reductase protein. In the present study, digestion of the highly purified diaphorase protein is shown to result in a soluble fully functional protein fragment, catalyzing both NADPH dependent cytochrome *c* and INT reduction. These results indicate that the soluble INT and cytochrome *c* reductase activity liberated from the plasma membrane by papain digestion originated from the membrane bound cytochrome *c* reductase protein.

2. Materials and methods

2.1. Materials

Papain, L-*trans*-epoxysuccinyl-leucylamide(4-guanidino)butane (E-64), papain attached to agarose, iodonitrotetrazolium violet (INT), octylglucoside (OG), cytochrome *c* (horse heart, type III), superoxide dismutase (SOD), guanosine 5′-[γ-thio] triphosphate (GTPγS), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF) and 2′,5′-ADP agarose were purchased from Sigma Chemical Co. The silver staining kit was purchased from BioRad.

2.2. Preparation of a partially purified cytochrome b_{558} and a highly purified cytochrome c reductase from the plasma membrane of bovine neutrophils

Neutrophils were isolated from bovine blood by isotonic lysing of red cells and gradient centrifugation as previously described [15]. Membrane fractions were prepared by differential centrifugation following ultrasonic disruption of neutrophils. Briefly the neutrophils were disrupted by five 15 s sonication bursts at 40% output from a microsonication probe (Heat Systems, model 350) at 0–4°C. Unbroken cells, nuclei, granules and other cellular debris were removed

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Abbreviations: SDS, sodium dodecyl sulfate; INT, *p*-iodonitrotetrazolium violet; OG, *n*-octyl glucoside; GTPγS, guanosine 5′-[γ-thio]-triphosphate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycolbis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; FAD, flavin-adenine dinucleotide; SOD, superoxide dismutase

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from the sonicate by centrifugation at 10 000×*g* for 10 min at 4°C in a Sorvall ss 34 rotor. The post nuclear supernatant was then subjected to ultracentrifugation at 120 000×*g* for 1 h at 4°C to separate plasma membranes (pellet) from cytosol (supernatant). The pellet was washed with 1 M NaCl by centrifugation, resuspended in buffer A containing 1 mM PMSF, and used directly as the membrane fraction. The membrane fraction was solubilized with buffer B (50 mM sodium phosphate, pH 7.4, 40 mM octylglucoside, 1 mM EGTA, 0.5 mM D TT, 1 mM NaN_3, 20% glycerol (v:v), 1 mM PMSF, 2 μg/ml leupeptin and 5 μg/ml pepstatin A). The OG solubilized membranes (2 mg/ml) were mixed for 2 h at 4°C with 0.1 volume of DEAE-Sepharose pre-equilibrated with buffer B. The suspension was then packed into a 1.2×10 cm column and the unbound protein collected and combined with two bed volume washes of buffer B. This resulted in the separating of cytochrome b_{558} (not bound) from the bound cytochrome *c* reductase [15]. The non-bound protein from the DEAE-Sepharose treated solubilized plasma membranes, containing all the cytochrome b_{558}, was incorporated into phospholipid liposomes essentially as reported by Koshkin [18] and used following gel filtration [15].

Following washing the DEAE-Sepharose with buffer B (20 ml) containing 100 mM NaCl the cytochrome *c* reductase activity was eluted with buffer B (20 ml) containing 250 mM NaCl. The 250 mM NaCl elute from the DEAE-Sepharose (8.3 mg protein in 10 ml) was first diluted with buffer B containing 30 mM OG to reduce the salt concentration to 100 mM and then applied to a 2′,5′-ADP agarose affinity column (0.8×3.5 cm) pre-equilibrated with buffer B (containing 30 mM OG) at a flow rate of 10 ml/min. Following washing of the column with 25 ml buffer B the cytochrome *c* reductase was eluted with buffer B (containing 30 mM OG) supplemented with 1 mM NADPH.

2.3. Proteolytic digestion of neutrophil membranes with papain

The plasma membrane fraction (100 μg protein) prepared as previously described [15], was suspended in buffer A (10 mM potassium phosphate, 150 mM NaCl, pH7.4) at 5–10 mg/ml and treated with 0.5–2 μg (0.005–0.02 units) of papain per 100 μg membrane protein for 10 min at 4°C. Papain digestion was terminated by the addition of E-64 at a 4:1 (w/w) ratio of papain to E-64.

When insoluble papain (papain attached to agarose) was utilized, it was first equilibrated with buffer A supplemented with 10 mM DTT for 30 min at 4°C and washed with buffer A four times by centrifugation (10 000×*g* for 10 min).

2.4. Separation of the papain digested membrane from the components of the membrane released by papain digestion

The membrane components released by papain digestion were separated from the membranes utilizing a 15–50% discontinuous sucrose gradient (volume, 8 ml). Each gradient layer (15%, 30%, 40% and 50% sucrose) was 2 ml in volume and centrifugation of the gradient was at 107 000×*g* for 2 h at 4°C. Non-digested membranes were also applied to a sucrose gradient together with E-64 inhibited papain to serve as a control. Following centrifugation, the supernatant and each gradient layer were collected and analyzed for protein and enzymatic activity.

2.5. Enzymatic activity

Superoxide generation by the partially purified cytochrome b_{558} incorporated into liposomes was achieved by activating 20 μg protein of the cytochrome b_{558} liposome preparation with 100 μM SDS in the presence of 1 μM FAD, 1 μM GTPγS and a saturating amount of cytosol (10 μg of cytosolic protein/μg of membrane protein) for 5 min at 25°C in a volume of 750 μl [15]. The reaction assay buffer 250 μl (400 μM cytochrome *c*, 4 mM NaN_3, 4 mM Mg^{2+}, 10 mM phosphate buffer pH 7.4) was then added and the 1 ml sample aliquoted equally into two cuvettes. Superoxide production was initiated by addition of 200 μM NADPH (10 μl of 0.01 M) and evaluated as SOD sensitive cytochrome *c* reduction [15].

INT and cytochrome *c* reductase activities were measured by monitoring the absorption increase of reduced INT at 500 nm and of ferrocytochrome *c* at 550 nm, and quantified using extinction coefficients of 11 mM^{-1} cm^{-1} for INT [19] and 21 mM^{-1} cm^{-1} for ferrocytochrome *c*. The assay mixture contained, in addition to the protein preparation, 50 μM INT or 125 μl of reaction assay buffer (100 μM cytochrome *c*, 1 mM NaN_3, 1 mM Mg^{2+}, 10 mM phosphate buffer pH 7.4), 150 units of SOD and 200 μM NADPH in a total volume of 500 μl. Buffer A was used as a reference.

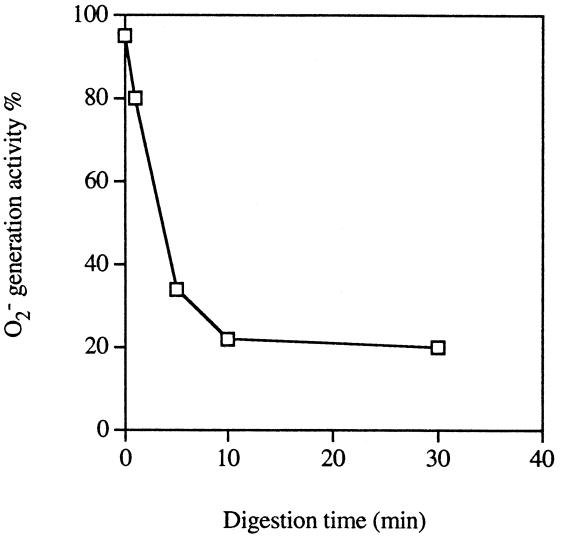

Fig. 1. Superoxide generating activity of plasma membranes as a function of papain digestion time. Neutrophil plasma membranes were incubated with papain (0.02 units per 100 μg membrane protein) at 4°C. At the specified time indicated, an aliquot was withdrawn and the papain inhibitor E-64 added as described in Section 2. Superoxide generation was evaluated in the reconstitution cell-free system as detailed in Section 2. The control superoxide generation was 101.4 nmol/min/mg protein.

3. Results

3.1. Papain digestion of neutrophil plasma membrane proteins

Papain digestion of the plasma membrane of bovine neutrophils was carried out by incubating papain together with the non-active membrane at 25°C. At specified digestion times, an aliquot of the sample was withdrawn and quenched with papain inhibitor E-64. Superoxide generating activity of the treated sample was evaluated in the cell-free activation system containing saturating levels of cytosol [15]. As demonstrated in Fig. 1, superoxide generation of these samples decreased as a function of papain digestion time, indicating that a membrane component of the NADPH oxidase is sensitive to proteolysis. The zero time sample was prepared with E-64 inhibited papain. A control which did not contain papain had an activity identical to the zero time sample (data not shown).

3.2. Separation of the papain released soluble component from the papain digested membranes

Our initial experimental design was aimed at investigating whether controlled proteolysis of membrane bound cytochrome b_{558} would result in formation of a soluble fragment of the cytochrome with an intact NADPH/FAD active site believed to be external to the membrane surface [20]. Our previous studies [15] pointed out that an INT reductase activity is associated with cytochrome b_{558} and that it could represent a part of the electron transfer reaction responsible for transferring electrons from NADPH, to FAD, heme and finally to molecular oxygen [16]. A fragment of cytochrome b_{558} could then potentially still possess INT reductase activity even

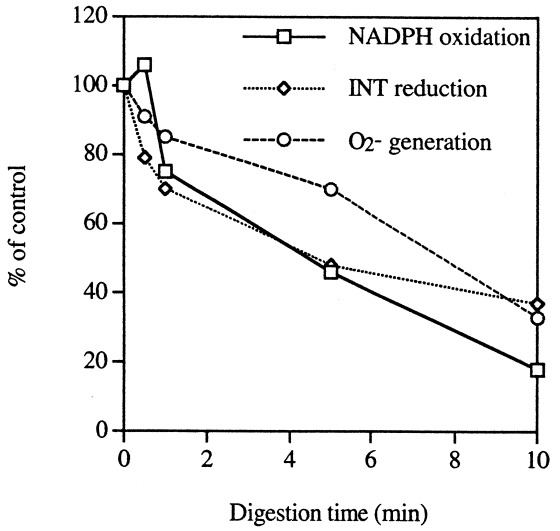

Fig. 2. Superoxide generation, NADPH oxidation and INT reductase activity profiles of papain digested plasma membranes as a function of the time of digestion. The results shown are the percent change from the control, non-papain treated preparation. The papain concentration was 0.005 units/100 μg membrane protein. E-64 (0.125 μg) was added to quench the digestion at appropriated time intervals. Control activities: NADPH oxidation, 547 nmol/min/mg protein; superoxide generation, 291 nmol/min/mg protein; INT reduction, 585 nmol/min/mg protein.

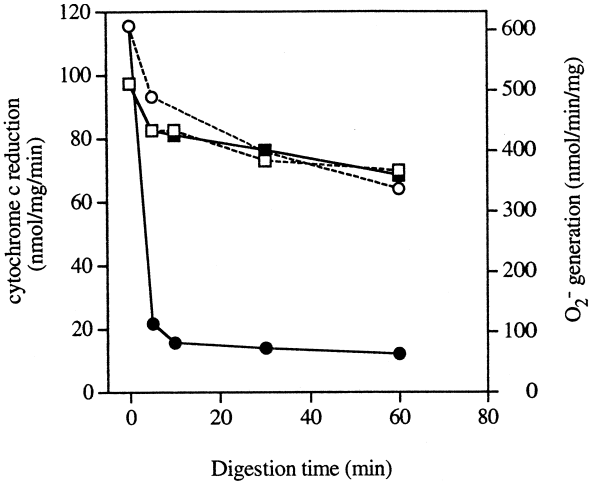

Fig. 3. The effect of papain digestion on O_2^- generation (cytochrome b_{558} liposome) and cytochrome *c* reduction (partially purified cytochrome *c* reductase). The cytochrome b_{558} liposomes (100 μg, circles) or cytochrome *c* reductase eluted from the DEAE-Sepharose column (100 μg, squares) were incubated separately with 2 units of insoluble papain (closed symbols) or 2 units of insoluble papain together with 25 μg E-64 (control, open symbols) in a total volume of 200 μl. The mixtures were gently shaken at 25°C. At the indicated time, 40 μl of the mixture was withdrawn and centrifuged at 10 000×*g* for 10 min (5 μg of E-64 was added to the papain treated preparation). The supernatants were assayed for activity as detailed in Section 2.

though the preparation had lost superoxide generating capacity.

To examine this possibility, we separated the papain released components (supernatant) from the digested membranes by means of a sucrose gradient and evaluated the supernatant's capability of catalyzing NADPH oxidation, INT reduction and SOD sensitive and insensitive cytochrome *c* reduction. The SOD sensitive cytochrome *c* reduction is taken to represent superoxide generation. A control non-digested membrane sample (treated with E-64 inhibited papain) was carried out in parallel. The control supernatant had no significant NADPH oxidation activity and was not able to reduce INT. On the other hand, the supernatant containing the soluble components released by papain digestion had a significant amount of NADPH activity in that it was able to catalyze the NADPH dependent reduction of INT and an SOD insensitive reduction of cytochrome *c* (Table 1). The separated papain treated membranes showed a marked decrease in the production of superoxide in comparison to the control, non-digested membranes. These results indicate that an enzymatically active component was liberated from the membrane by the papain digestion. Based on the finding of two distinguished INT reductases existing in the plasma membrane [15], we traced the origin of the 'solubilized' active component (below).

3.3. *Susceptibility of the O_2^- generating oxidase and the cytochrome c reductase to papain digestion*

Papain digestion of resting membranes was repeated at a lower papain concentration (0.005 units/100 μg of membrane). After digestion for appropriate time intervals (0, 0.5, 1, 5 and 10 min) the reaction was terminated with E-64 and assayed spectrophotometrically for superoxide production (SOD sensitive cytochrome *c* reduction at 550 nm), NADPH oxidation at 340 nm and INT reduction at 500 nm. All three of the activities decreased as a function of digestion time with papain (Fig. 2).

Separation of cytochrome b_{558} from the SOD insensitive cytochrome *c* reductase activity can be achieved by means of DEAE-anion exchange chromatography [15]. The partially purified cytochrome b_{558} liposome preparation and cytochrome *c* reductase were separately subjected to papain digestion and were analyzed for activity (Fig. 3). Controls were those in which papain was added together with its inhibitor E-64. From Fig. 3, it is clear that superoxide generation by cytochrome b_{558} is very sensitive to papain digestion (closed

Table 1
Enzymatic activities catalyzed by the papain released soluble component from neutrophil plasma membranes

Activity analyzed	Additional components added to the assay	Activity catalyzed by the supernatant from papain treated membranes (nmol/min/mg protein)
INT reduction	INT, NADPH, FAD	135 ± 17
	INT, NADPH	110
	INT	0
Cytochrome *c* reduction	Cytochrome *c*, FAD,NADPH, SOD	250 ± 20
	Cytochrome *c*, FAD, NADPH	250 ± 20

See Section 2 for assay procedures and concentration of reagents.

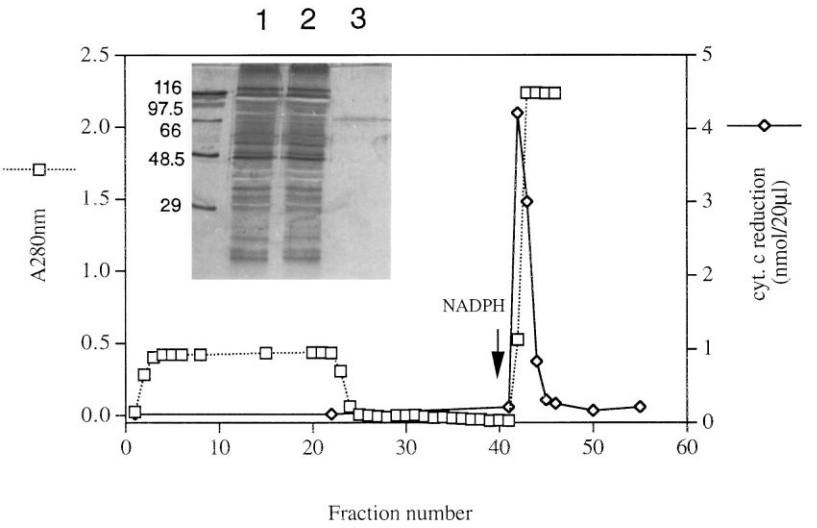

Fig. 4. Purification of the NADPH dependent cytochrome *c* reductase by 2′,5′-ADP agarose affinity chromatography. The 250 mM NaCl eluate from DEAE-Sepharose chromatography (8.3 mg protein) was applied to the 2′,5′-ADP agarose column and the cytochrome *c* reductase eluted with 1 mM NADPH as described in Section 2. The insert is a silver stained 12% SDS-PAGE of the column samples. Lane 1, the preparation prior to affinity column; lane 2, the column flow through fraction; lane 3, the NADPH eluate (fraction 42). Molecular standards are indicated on the left side of the gel.

circles) while the activity of the SOD insensitive cytochrome *c* reductase is not affected by papain (closed squares) when compared with corresponding controls (open squares). No cytochrome *c* reductase activity (SOD insensitive) could be detected in the cytochrome b_{558} liposome preparation. These results suggest that the active component released by papain digestion from the membrane catalyzing an NADPH dependent INT reductase could not be a resultant of cytochrome b_{558} cleavage but must originate from the cytochrome *c* reductase protein.

3.4. Purification and digestion of the NADPH dependent cytochrome c reductase

To further verify our hypothesis, the cytochrome *c* reductase protein was purified more than 150-fold by 2′,5′-ADP agarose affinity chromatography (Fig. 4). Purified reductase showed a single band with an apparent molecular mass of 75 kDa on SDS-PAGE (Fig. 4 insert, lane 3). The specific activity of the purified enzyme was 10.1 μmol/min/mg for cytochrome *c* reduction and 4.77 μmol/min/mg for INT reduction. No cytochrome *c* or INT reductase activity was observed in the flow through fractions. The copurification of the cytochrome *c* reductase and INT reductase activities confirm the 75 kDa protein to be both an NADPH dependent INT reductase as well as an NADPH-cytochrome *c* reductase. Both enzymatic activities are absolutely dependent on NADPH (data not shown).

Since the enzymatic activity of the NADPH-INT diaphorase of the partially purified NADPH cytochrome *c* reductase is insensitive to papain digestion (Fig. 3) and papain digestion of the plasma membrane releases a soluble cytochrome *c* reductase from the membrane capable of NADPH dependent cytochrome *c* reduction (Table 1), we examined the possibility that the purified enzyme undergoes proteolysis without enzymatic modification. Fig. 5 shows that upon controlled papain digestion the 75 kDa diaphorase was cleaved into peptides of 65 kDa, 53 kDa, 21 kDa and 22 kDa while the enzymatic activity was not modified. The 21 kDa and 22 kDa bands were not due to a contamination of papain (which has a M_r of 23 kDa) since this doublet did not disappear upon removing the insoluble papain from the sample by centrifugation and filtration through a 0.2 μm filter (Gelman, Acrodisc CR PTFE) (Fig. 5). A control with insoluble papain in the absence of the diaphorase protein showed no papain band on SDS-PAGE following removal of the insoluble papain by filtration. It is concluded that with limited digestion there are at least two papain digestion sites on the diaphorase accounting for the peptide banding pattern observed. The fact that pa-

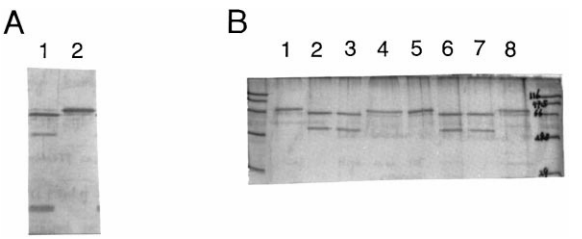

Fig. 5. SDS-PAGE of the purified cytochrome *c* reductase following digestion with insoluble papain. The purified cytochrome *c* reductase (2.2 μg in 110 μl) was digested with increasing amounts of insoluble papain (0, 0.2 and 1 unit) at 25°C. At the indicated digestion time, an aliquot (50 μl) was withdrawn followed by addition of 10 μg E-64 (5 μl) and centrifuged at 10 000×*g* for 10 min. The supernatants were assayed for cytochrome *c* reduction and analyzed by SDS-PAGE. A: Silver stained 10% SDS-PAGE of preparations in which insoluble papain (1 unit) was removed by centrifugation and the sample filtered through a 0.2 μm filter (Gelman, Acrodisc CR PTFE) prior to sample application to the gel: lane 1, 10 min papain digestion; lane 2, digestion with E-64 inhibited papain. It is clear that the preparative treatment prior to application of the sample to the gel completely removes the papain from the samples. B: Silver stained SDS-PAGE (10%). Lanes 1–4, 10 min digestion; lanes 5–8, 60 min digestion time. Lanes 1 and 5, no papain; lanes 2 and 6, 0.2 units; lanes 3 and 7, 1 unit; and for lanes 4 and 8, E-64 (10 μg) inhibited papain (1 unit) replaced the active enzyme. The corresponding cytochrome *c* reductase activity (in nmol/min/mg protein) of those samples in panel B were from lanes 1–8: 5.82, 8.60, 8.07, 5.69, 7.28, 8.73, 8.07, 5.03.

pain is able to generate a soluble and fully functional NADPH dependent INT and cytochrome *c* reductase (65–53 kDa protein) from its membrane bound form (75 kDa) indicates that the papain digestion cleaves at a protein membrane anchor remote from its active site.

4. Discussion

In this study we have purified an NADPH dependent cytochrome *c* reductase from resting bovine neutrophil membranes which catalyzes as well an NADPH dependent INT reductase activity. The 75 kDa protein is readily digested by the protease papain into a major 65 kDa (or 53 kDa) and the minor 21 kDa and 22 kDa peptides without loss in enzymatic activity. Preliminary experiments which subjected the membrane preparation used in our experiments to centrifugation through a discontinuous sucrose gradient (30, 40 and 50% sucrose) separated the membrane fraction into two major components, one equilibrating above 30% sucrose and the other above the 40% sucrose layer. The membrane band above the 30% sucrose layer (plasma membranes) had 31% of the total protein associated with 76% of the total cytochrome *c* reductase activity. The membrane banding above the 40% sucrose (granular fraction) represents 28% of the total protein with 25% of the cytochrome *c* reductase activity. It would appear that quantitatively the plasma membrane contains the major proportion of that activity designated as NADPH-cytochrome *c* reductase.

Using a highly purified intact NADPH cytochrome *c* reductase from the membrane fraction of bovine neutrophils we have confirmed the previous conclusion that this enzyme is as well an INT reductase. The intact enzyme did not promote a stimulation of O_2^- production by either reflavinated or non-flavinated cytochrome b_{558} liposomes in the cell-free system (data not shown) indicating that this membrane oxidoreductase is not likely to be involved in the superoxide generation catalyzed by the NADPH oxidase complex. The data in Fig. 5 provide direct evidence for the susceptibility of this 75 kDa reductase protein towards papain proteolysis with the digested enzyme retaining its catalytic activity. Although a number of NADPH-cytochrome *c* reductase's biochemical and immunological properties have a resemblance to the liver microsomal NADPH-cytochrome P-450 proteins [10,13], the in vivo function of this leukocyte cytochrome *c* reductase is currently unknown. The availability of a highly purified preparation will assist in the evaluation of the function of this enzyme in the leukocyte.

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