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CHARACTERIZATION OF MULTIPLE ACTIVE FORMS OF THE NADPH DEHYDROGENASE COMPONENT OF THE OXIDASE COMPLEX FROM RABBIT PERITONEAL NEUTROPHILS BY PHOTOLABELING WITH AN ARYLAZIDO DERIVATIVE OF $NADP^+$

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Summary: A NADPH cytochrome c oxidoreductase purified from membranes of rabbit peritoneal neutrophil was shown to behave as the NADPH dehydrogenase component of the $O_2^{\cdot-}$ generating oxidase complex. A photoactivable derivative of $NADP^+$, azido nitrophenyl-γ-aminobutyryl $NADP^+$ (NAP_4-$NADP^+$), was synthesized in its labeled [^{3}H] form and used to photolabel the NADPH cytochrome *c* reductase at different stages of the purification procedure. Control assays performed in dim light indicated that the reduced form of NAP_4-$NADP^+$ generated by reduction with glucose-6-phosphate and glucose-6-phosphate dehydrogenase was oxidized at virtually the same rate as NADPH. Upon photoirradiation of the purified reductase in the presence of [^{3}H]NAP_4-$NADP^+$ and subsequent separation of the photolabeled species by sodium dodecyl sulfate polyacrylamide gel electrophoresis, radioactivity was found to be present predominantly in a protein band with a molecular mass of 77-kDa and accessorily in bands of 67-kDa and 57-kDa. Evidence is provided that the 67-kDa and 57-kDa proteins arose from the 77-kDa protein by proteolysis. Despite removal of part of the sequence, the proteolyzed proteins were still active in catalyzing electron transport from NADPH to cytochrome *c* and in binding the photoactivable derivative of $NADP^+$.

The component of the respiratory burst oxidase which reacts with NADPH is a flavoprotein (for review, see 1,2) which most likely functions to transfer electrons from NADPH to a low-potential *b*-type cytochrome (3). Rabbit peritoneal neutrophils contain a flavin-dependent NADPH cytochrome *c* reductase of 77-kDa which can be extracted and purified in the presence of detergent. This reductase behaves as the NADPH dehydrogenase component of the $O_2^{\cdot-}$ generating oxidase. During the course of its purification, limited proteolysis occurred, resulting in the accumulation of water-soluble proteins of

Abbreviations: **DFP:** diisopropyl fluorophosphate ; **NAP_4NADP:** azidonitrophenyl-γ-aminobutyryl NADP ; **SDS-PAGE:** sodium dodecyl sulfate polyacrylamide gel electrophoresis ; **PBS:** phosphate buffer saline consisting of 2.7 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, PH 7.4 ; **EMP:** medium consisting of 20% ethylene glycol (w/v), 0.1 M mannitol and 10 mM Na phosphate, pH 7.4 ; **EMPT:** EMP supplemented with 0.2% Triton X-100.

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67-kDa and 57-kDa which exhibited nearly the same specific activity as the native enzyme. The specific recognition of NADPH by the substrate binding site of the enzyme was explored by the technique of photolabeling, using an arylazido derivative of $NADP^+$.

MATERIALS AND METHODS

Ferricytochrome *c* (horse heart, grade VI), arachidonic acid, oyster glycogen type II, diisopropyl fluorophosphate, Triton X-100, superoxide dismutase were purchased from Sigma, NADPH and NADH from Boehringer and 2'-5' ADP Sepharose from Pharmacia. Neutrophils were collected from rabbit peritoneal cavities 12 h after injection of 300 ml of sterile 0.9% NaCl solution containing 0.1% oyster glycogen. [^{3}H]Azidonitrophenyl-γ-aminobutyryl-$NADP^+$ (NAP_4-$NADP^+$) (Figure 1) was synthesized as described in (4) by a procedure derived from that described in (5). Its specific radioactivity was 50.10^6 dpm/μmol. NAP_4-$NADP^+$ could be readily reduced in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

NADPH cytochrome *c* reductase activity was measured at 25°C in phosphate buffer saline (PBS) in the presence of 100 μM NADPH and 100 μM cytochrome *c*. Reduction of cytochrome *c* was followed at 550 nm. The absorption coefficient used was 21.1 $mM^{-1}.cm^{-1}$.

A membrane fraction enriched in NADPH cytochrome *c* reductase activity was obtained by differential centrifugation of a rabbit neutrophil homogenate obtained by sonication in the presence of 1 mM DFP. All operations were conducted at 2-4°C. After elimination of unbroken cells, large cell debris, and part of lysosomal granules by centrifugation at 2500g for 10 min, the supernatant was recovered and centrifuged at 140000g for 1 h. The pellet was resuspended in EMP medium supplemented with 0.4% sodium deoxycholate and 0.2% Triton X-100, and used for extraction of the NADPH cytochrome *c* reductase by detergent and its purification. In brief, the membrane lysate was centrifuged at 140000g for 1 h. The NADPH-cytochrome *c* reductase present in the supernatant was purified by chromatography on a DE 52 cellulose column, followed by chromatography on a 2'-5'-ADP Sepharose column. Both columns were equilibrated in EMPT. The DE 52 cellulose column was eluted with a 0-0.25 M sodium sulfate

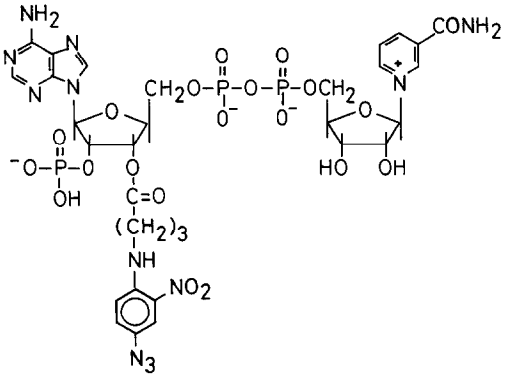


Figure 1. Structure of arylazido $NADP^+$ (NAP_4-NADP).
The position of the photoactivable residue is given by analogy with that reported for a photoactivable derivative of NAD^+ (5).

gradient in EMPT. A major peak of reductase activity referred to as DE I was eluted at 0.9-0.11 M sodium sulfate, followed by a minor one termed DE II, recovered at 0.14-0.15 M sodium sulfate. The content of the DE I peak and that of the DE II peak were subjected separately to chromatography on 2'-5'-ADP Sepharose. After washing with EMPT, a solution of 1 mM NADPH in EMPT was applied to the column, resulting in the elution of a virtually homogeneous NADPH cytochrome *c* reductase as judged by SDS-PAGE, with a specific activity of 4 to 8 µmol of cytochrome *c* reduced x min^{-1} x mg $protein^{-1}$ (for further details, see F. Laporte, J. Doussière, P.V. Vignais, Biochem. Biophys. Res. Commun., in Press).

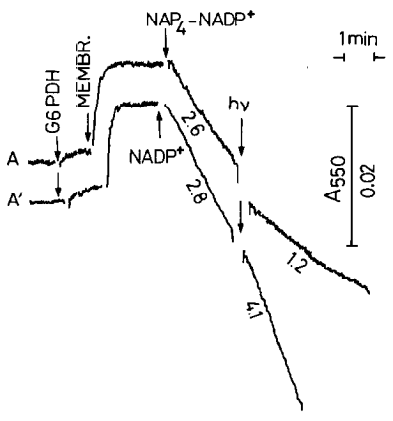
NAP_4-$NADP^+$ was readily reduced in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase, as detailed in Results. Photoirradiation with [^{3}H]NAP_4-NADP(H) was performed with a Xenon XBO 1000W HS lamp (Müller GmbH, Moosinning FRG) (4). Photolabeled proteins were separated by SDS-PAGE (6). The gels were silver stained (7) and cut into 2 mm pieces, which were digested in 10% H_2O_2 at 60°C overnight. Excess H_2O_2 was eliminated by the addition of catalase. The radioactivity of each digest was measured by liquid scintillation (8).

RESULTS

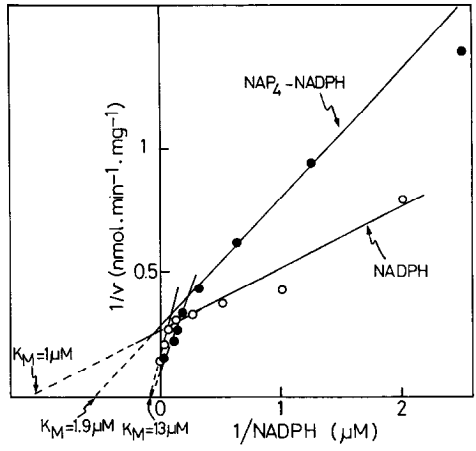
Capacity of NAP_4-NADPH to be oxidized by the NADPH cytochrome *c* reductase of rabbit neutrophils

As illustrated in Figure 2, the reduced forms of $NADP^+$ and NAP_4-$NADP^+$ generated in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase were oxidized virtually at the same rate by a membrane preparation of rabbit neutrophils. Photoirradiation resulted in about 50% inhibition of the rate of oxidation of NAP_4-NADPH ; on the other hand, oxidation of NADPH was slightly accelerated, suggesting the participation of flavins, since reduced flavins are susceptible to photooxidation (9). NADPH added in large excess to the photolabeled membranes did not restore the lost NADPH cytochrome *c* reductase activity (data not shown), consistent with the covalent modification of the enzyme by the nitrene derivative of NAP_4-NADPH, and the resulting inactivation of the reductase.

A further control aimed at exploring specific interactions consisted in analyzing the kinetics of reduction of cytochrome *c* by NADPH or NAP_4-NADPH in the presence of rabbit neutrophil membranes, in dim light. The double reciprocal plots of the rate of cytochrome *c* reduction as a function of the concentration of NADPH or NAP_4-NADPH were curvilinear (Figure 3). Similar plots were obtained with purified NADPH cytochrome *c* reductase (not shown). The plots were decomposed into two rectilinear portions corresponding to the high and low affinity components of cytochrome *c* reductase activity, from which Vmax and K_M values could be calculated. The



(2)



(3)

Figure 2. Oxidation of NAP_4-NADPH and NADPH by rabbit neutrophil membranes in the presence of cytochrome *c*.
The photometric cuvette thermostated at 25°C contained 240 μg of rabbit neutrophil membranes suspended in 2 ml of PBS, and permeabilized with 0.05% sodium deoxycholate, and 100 μM cytochrome c. NAP_4-$NADP^+$ and $NADP^+$ were added at the final concentration of 10 μM and were continuously reduced with 1 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase. After a few min of cytochrome *c* reduction, the suspension in the photometric cuvette was subjected to a 30 s-photoirradiation with a 1000 W lamp placed at 30 cm from the cuvette. The number on the traces refer to the rates of cytochrome *c* reduction in nmol x min^{-1} x mg $protein^{-1}$.

Figure 3. Double-reciprocal plots of the rate of NADPH cytochrome *c* reductase *vs* NADPH or NAP_4-NADPH concentrations.
The photometric cuvette thermostated at 25°C contained 350 μg of rabbit neutrophil membrane protein suspended in 2 ml of PBS in the presence of 0.05% sodium deoxycholate, 100 μM cytochrome *c*, and increasing concentrations of NADPH or NAP_4-NADPH (obtained by reduction with 1 mM glucose-6-phosphate and glucose 6-phosphate dehydrogenase).

high affinity component was characterized by K_M values of 1 μM for NADPH and 1.9 μM for NAP_4-NADPH and a Vmax of 4 nmol cytochrome *c* reduced x min^{-1} x mg $protein^{-1}$ for both electron donors. The low affinity component exhibited for NADPH and NAP_4-NADPH the same K_M values (13 μM) and Vmax values (9 nmol cytochrome *c* reduced x min^{-1} x mg $protein^{-1}$). These results indicate that, in the absence of photoirradiation, NADPH and NAP_4-NADPH are equally efficient as electron donors for the NADPH cytochrome *c* reductase of rabbit neutrophil membranes.

Photolabeling of rabbit neutrophil membranes and purified NADPH cytochrome *c* reductase by [^{3}H]NAP_4-$NADP^+$

Upon chromatography of a detergent extract of rabbit neutrophil membranes on DE 52 cellulose, two peaks of NADPH cytochrome *c* reductase activity were resolved which were referred to as DE I and DE II. The reductase activity contained in the main peak DE I was further purified by affinity chromatography on 2'-5' ADP Sepharose. The crude detergent extract, the DE I and DE II fractions

and the 2'-5' ADP Sepharose fraction were photoirradiated in the presence of [^{3}H]NAP$_4$-NADP$^+$ and the labeled proteins were subjected to SDS-PAGE. As shown by the profile of radioactivity in Figure 4A, photoirradiation of the crude extract resulted in photolabeling of a large number of proteins. In the purified DE I and DE II fractions (Figures 4B and 4C), the number of labeled proteins was reduced to essentially three protein species with molecular masses of 77-kDa, 67-kDa and 57-kDa. The predominantly labeled protein band

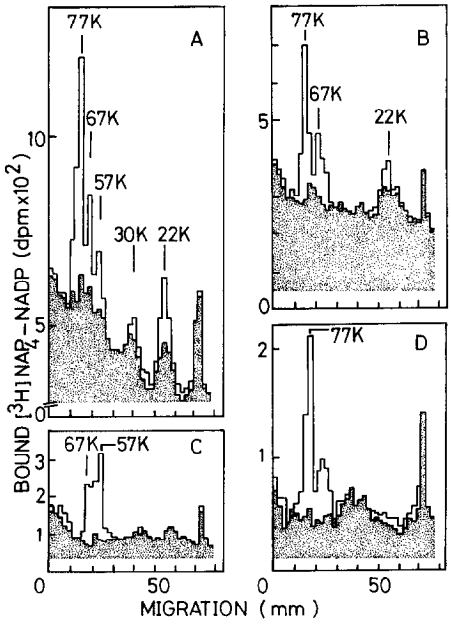


Figure 4. Photolabeling by [^{3}H]NAP$_4$-NADP$^+$ of crude and purified fractions from a rabbit neutrophil extract characterized by a NADPH-cytochrome *c* reductase activity. [^{3}H]NAP$_4$-NADP$^+$ (20 µM final concentration 50000 dpm/nmol) was incubated for 5 min in the dark with protein fractions recovered at different stages of the purification procedure and corresponding to **(A)** the detergent extract of rabbit neutrophil membranes (300 µg protein), **(B)** the DE I peak from the DE 52 cellulose column (120 µg protein) ; **(C)** the DE II peak from the DE 52 cellulose chromatography column (110 µg protein) ; **(D)** the purified dehydrogenase eluted from 2'-5' ADP Sepharose with 1 mM NADPH (17 µg). In the latter case, free NADPH was removed by gel filtration on an AcA 202 column, equilibrated in the EMPT medium. Parallel incubations in the dark were performed, in which the protein fractions were first incubated with an excess of NADPH (1 mM) for a few min prior to addition of [^{3}H]NAP$_4$-NADP$^+$. In all cases the dilution medium was PBS (final volume 1 ml). This was followed by 3 cycles of photoirradiation of 10 s each with a 1000 W lamp (cf. Materials and Methods). After photoirradiation, 1 ml of PBS was added to each tube, and proteins were denaturated by 0.1 N perchloric acid. Proteins were recovered by centrifugation, rinsed with cold acetone, dissolved in the Laemmli medium, and separated by SDS-PAGE using 12% acrylamide gels. The gels were silver-stained and then cut into 2 mm pieces which were counted for radioactivity as described in Materials and Methods. The open bars correspond to the covalently bound [^{3}H]NAP$_4$-NADP$^+$ in the absence of added NADPH. The dotted bars correspond to the incorporated [^{3}H]NAD$_4$-NADP$^+$ in the presence of NADPH.

in the DE I fraction was a band of 77-kDa molecular mass (Figure 4B). Depending on the enzyme preparation, the amount of incorporated radioactivity in the 57-kDa band and more particularly in the 67-kDa band varied, and labeled bands of intermediary molecular masses were often observed. The 67-kDa and 57-kDa labeled species were predominant in peak DE II (Figure 4C). The photolabeling was specific because addition of an excess of NADPH (1 mM) prior to photoirradiation was able to decrease markedly the covalently incorporated radioactivity. The final step of purification on 2'-5' ADP Sepharose yielded a protein which after photoirradiation exhibited a radioactivity profile similar to that observed with the photolabeled DE I fraction, consisting of a major labeled band with a molecular mass of 77-kDa, and minor ones with molecular masses of 67-kDa and 57-kDa (Figure 4D). Here again, the specificity of labeling by [^{3}H]NAP_4-$NADP^+$ was ascertained by prevention of the labeling upon addition of an excess of NADPH (1 mM) prior to photoirradiation.

When the DE I and DE II fractions were chromatographed on 2'-5'-ADP Sepharose, three protein bands, with molecular masses of 77-kDa, 67-kDa and 57-kDa, were revealed by silver staining, coinciding with the three photolabeled protein species described above (not shown). The 77-kDa protein was predominant after 2'-5'-ADP Sepharose chromatography of the DE I peak, whereas the 67-kDa protein and mostly the 57-kDa protein were in large excess after 2'-5'-ADP Sepharose chromatography of the DE II peak. Determination of the enzymatic activity did not reveal substantial differences between the three protein species. In brief, although the proteins identified by photolabeling differed by the size, they all were active in catalysis of NADPH oxidation, which is consistent with the presence of a NADPH binding site in all of them.

Different protein profiles were obtained at the stage of DE 52 chromatography, according to whether the antiprotease, DFP, was omitted or added to the neutrophil homogenate. When the homogenate was supplemented in DFP, the size of the DE I peak was much larger than that of the DE II peak. The DE II peak increased when DFP was omitted. In other words, proteolysis of the 77-kDa protein was able to yield smaller size proteins still retaining a substantial reductase activity, and a functional NADPH binding site.

DISCUSSION

The NADPH cytochrome *c* reductase activity of rabbit peritoneal neutrophils probed by a photoactivable derivative of $NADP^+$ most

likely reflects the NADPH flavodehydrogenase component of the $O_2^{\cdot-}$ generating oxidase (see Introduction). This membrane-bound enzyme required detergent for solubilization. Routinely, prior to purification, the neutrophil membranes were supplemented with DFP ; in this case, a predominent protein with a molecular mass of 77-kDa endowed with high NADPH cytochrome *c* reductase activity was recovered. This protein was specifically photolabeled with NAP_4-$NADP^+$. When DFP was omitted, proteolysis occurred, which generated two major soluble proteins with molecular masses of 67-kDa and 57-kDa from the detergent soluble 77-kDa protein. The water-soluble proteolyzed forms of the 77-kDa protein still exhibited a NADPH cytochrome *c* reductase activity and were able to bind NAP_4-$NADP^+$. It has been postulated that, in a cell-free system of activation of the respiratory burst, elicitation of oxidase activity required the translocation of a cytosolic NADPH-binding protein to the plasma membrane, and it was inferred that this protein could be the NADPH dehydrogenase component of the oxidase (10). This soluble NADPH-binding protein was suggested to be a 67-kDa protein, which is lacking in a form of autosomal chronic granulomatous disease (11). We would like to propose that a protein with similar properties might arise from partial proteolysis of the membrane-bound NADPH-dehydrogenase.

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