Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 85-93

PHOTOLABELING OF A $O_2^{\cdot-}$ GENERATING PROTEIN IN BOVINE POLYMORPHONUCLEAR NEUTROPHILS BY AN ARYLAZIDO $NADP^+$ ANALOG

Jacques DOUSSIERE, François LAPORTE and Pierre V. VIGNAIS

Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex, France

Received July 11, 1986

Summary. A plasma membrane fraction of bovine polymorphonuclear neutrophils enriched in NADPH-dependent, $O_2^{\cdot-}$ generating oxidase activity, and a number of fractions solubilized in detergent, recovered during the course of the purification of this oxidase have been tested for their ability to react with radiolabeled N-4-azido-2-nitrophenyl aminobutyryl $NADP^+$ (arylazido $NADP^+$ or NAP_4-$NADP^+$). In the dark, NAP_4-$NADP^+$ and its reduced form, NAP_4-NADPH, were found to inhibit competitively the NADPH-dependent $O_2^{\cdot-}$ generating oxidase activity of the plasma membrane fraction of bovine neutrophils activated by phorbol myristate acetate. The nitrene derivative formed upon photoirradiation of NAP_4-NADP(H) bound covalently to different proteins of the plasma membrane. Photolabeling of these proteins was prevented by preincubation with an excess of NADPH. Photolabeling of a protein of 65000 Mr was decreased by omission of phorbol myristate acetate as activating agent of the respiratory burst in neutrophils or by addition of micromolar amounts of Cibacron Blue and mersalyl which are known to inhibit the production of $O_2^{\cdot-}$ by the plasma membrane fraction. During the course of the purification procedure, the 65000 Mr protein emerged as the preferentially photolabeled protein. These data, in agreement with previous findings concerning the purification of an NADPH-dependent, $O_2^{\cdot-}$ generating oxidase protein of Mr 65000 from bovine neutrophils (Doussière, J. and Vignais, P.V. (1985) Biochemistry **24**, 7231-7239), strongly suggest that a single protein of Mr 65000, located in the plasma membrane fraction of bovine neutrophils, is able to act both as an NADPH deshydrogenase and as an oxygen reductase to generate $O_2^{\cdot-}$.

During the course of the respiratory burst, activated polymorphonuclear neutrophils accumulate a large amount of the superoxide anion, $O_2^{\cdot-}$. The enzymatic system responsible for the respiratory burst is generally believed to be a complex consisting of several molecular species, including a flavoprotein, a **b**-type cytochrome and possibly ubiquinone (for review, see 1). In a recent paper (2), we have described the purification of an $O_2^{\cdot-}$ generating oxidase from the plasma membrane of bovine neutrophils ; this protein of Mr 65000 was able to reduce O_2 directly to $O_2^{\cdot-}$ in the presence of NADPH. The purification of this NADPH-dependent, $O_2^{\cdot-}$ generating oxidase did

Abbreviations: NAP_4-$NADP^+$ or arylazido $NADP^+$: N-4-azido-2-nitrophenyl-aminobutyryl $NADP^+$; PMA : phorbol myristate acetate ; SOD : superoxyde dismutase ; $O_2^{\cdot-}$: superoxyde radical.

0006-291X/86 $1.50

not preclude the existence of additional oxidases potentially effective in the respiratory burst. This problem was directly approached by photolabeling NADPH-dependent proteins recovered at different steps of the purification of the $O_2^{\cdot-}$ generating oxidase from a membrane fraction of activated bovine neutrophils enriched in this oxidase. The data obtained were consistent with the conclusion that the 65000 Mr protein is the major catalyst of the reduction of O_2 into $O_2^{\cdot-}$ by NADPH.

MATERIALS AND METHODS

Ferricytochrome c (horse heart grade VI), superoxide dismutase and phorbol myristate acetate (PMA) were from Sigma, $NADP^+$ and NADPH were from Boehringer. DE52 cellulose was from Whatman and sterile Percoll was from Pharmacia. Stock solutions of phorbol myristate acetate were made up to 1 mg/ml in dimethylsulfoxide. Mersalyl was from Sigma and Cibacron Blue from Pierce. [^{3}H]azidonitrophenyl-γ-aminobutyryl-$NADP^+$ (NAP_4-$NADP^+$) was synthesized from [^{3}H]azido nitrophenyl-γ-aminobutyric acid ([^{3}H]NAP_4) and $NADP^+$, using the same method as that described in (3) for the synthesis of arylazido β-alanine ATP, with the modifications introduced in (4). [^{3}H]NAP_4 was synthetized from 4-fluoro-3-phenylazide and [^{3}H]γ-aminobutyric acid. The coupling step between [^{3}H]NAP_4 and $NADP^+$ involved the use of carbodiimidazole (5). In short, [^{3}H]NAP_4 (5 mg) and carbodiimizole (20 mg) were dissolved in 1.2 ml of dimethylformamide (dried over molecular sieves). Forty mg $NADP^+$ in 4 ml water were added ; the pH was adjusted to 7, and stirring was continued overnight at 26°C. The products were separated by chromatography on 3 MM Whatman paper overnight in a solvent made of butanol, water and acetic acid (5/3/2). NAP_4 migrated with a Rf of 0.96, $NADP^+$ with a Rf 0.23, and NAP_4-$NADP^+$ with a Rf of 0.45. The yield of coupling with respect to [^{3}H]NAP_4 was between 20 and 25%. The specific radioactivity of [^{3}H]NAP_4-$NADP^+$ was about 50000 dpm/nmol. In some photolabeling experiments, the protein fraction in 1 ml of 2.5 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4 pH 7.4 (phosphate saline buffer) supplemented with [^{3}H]NAP_4-$NADP^+$ was introduced into a 10 ml flask which was rotated horizontally at 100 rev./min in an ice-bath and photoirradiated for 2 min with an OSRAM lamp of 250W placed at 10 cm above the tube. In other photolabeling experiments, photoirradiation was performed with a Xenon lamp XBO 1000W HS (Müller GmbH, Moosinning, RFA).

Neutrophils were prepared from bovine blood as detailed in recent papers (2, 6). Activation of neutrophils in phosphate-saline buffer was carried out using 0.3 µg PMA per mg neutrophil protein (2,6) at a temperature of 37°C for 2 min. The suspension of activated neutrophils was subjected to sonication and a membrane fraction, enriched in plasma membrane and in $O_2^{\cdot-}$ generating oxidase, was recovered by differential centrifugation between 10000g and 100000g (2). Solubilization of the oxidase activity by Triton X-100 from the 100000g membrane fraction, and purification of the 65000 Mr oxidase by DE52 cellulose chromatography followed by Sephadex G200 chromatography and isoelectric focusing were performed as previously described (2). Protein concentration was determined by the method of Bradford (7).

Prior to Na Dod SO_4 polyacrylamide gel electrophoresis, proteins in Triton X-100 were precipitated by cold 0.1N perchloric acid. The precipitate was washed with cold acetone and redissolved in 0.2M Na phosphate pH 7.5 and 1% Na Dod SO_4. After heat treatment for 2 min at 100°C and reduction by mercaptoethanol, proteins were analyzed by polyacrylamide gel electrophoresis in the presence of Na Dod SO_4 as described in (8).

The rate of generation of $O_2^{\cdot-}$ by NADPH oxidase was calculated from the difference between the rate of reduction of ferricytochrome **c** determined at 550 nm (ε_{550} 21 $mM^{-1}cm^{-1}$) in the absence and presence of superoxide dismutase. The assay was carried out at 25°C in the phosphate-saline medium in the presence of 100 µM cytochrome **c** and 200 µM NADPH, final volume 2 ml.

RESULTS

1. Spectral characteristics and photochemical reactivity of NAP_4-$NADP^+$

The spectrum of NAP_4-$NADP^+$ has maxima at 260 nm and 480 nm (Figure 1). The same peak at 480 nm, typical of the arylazido adduct, was reported for arylazido-β alanine NAD^+, a closely related compound (3). The molar extinction coefficient at 480 nm, 5.9×10^3, was used to calculate the concentration of NAP_4-$NADP^+$. When NAP_4-$NADP^+$ was supplemented with glucose 6-phosphate and glucose 6-phosphate dehydrogenase, an additional peak centered at 340 nm appeared, indicating that the $NADP^+$ moiety of the photoprobe had been reduced by glucose 6-phosphate (Insert, Figure 1). This was a convenient way to obtain the reduced form of NAP_4-$NADP^+$. Based on the incorporated radioactivity belonging to [^{3}H]γ-aminobutyric acid and the spectral data, a single arylazido residue was found to bind to $NADP^+$; similar data have been reported for arylazido-β-alanine NAD^+ (3). In addition, it has been demonstrated (3) by enzymatic and physical approaches that esterification of NAD^+ by arylazido-β-alanine occurs on the 3'-OH of

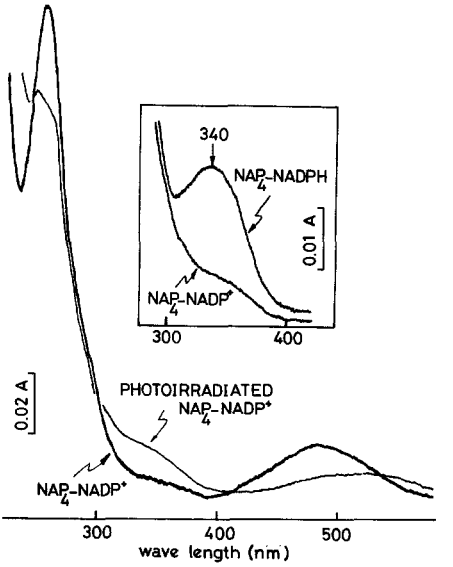

Figure 1. Absorption spectra of NAP_4-$NADP^+$ and NAP_4-NADPH. Effect of photoirradiation and reduction by glucose 6 phosphate in the presence of glucose 6 phosphate dehydrogenase (INSERT).

the ribose associated with the adenine portion of NAD^+ ; most probably the arylazido adduct binds at the same hydroxyl group in $NADP^+$.

Photoirradiation of NAP_4-$NADP^+$ was accompanied by disappearance of the peak at 480 nm (Figure 1).

2. Effect of NAP_4-$NADP^+$ on the $O_2^{\cdot-}$ generating oxidase activity

The $O_2^{\cdot-}$ production by the 100000g membrane fraction of activated bovine neutrophils was assayed by the SOD-inhibitable reduction of cytochrome **c** (Trace B-Figure 2). The first part of the assay was conducted in the absence of photoirradiation. NAP_4-NADPH generated from NAP_4-$NADP^+$ by glucose-6-phosphate and its specific dehydrogenase was not oxidized by the 100.000g membrane fraction of bovine neutrophils previously activated by PMA (Figure 2, Trace A), indicating that NAP_4-NADPH was not a substrate for the $O_2^{\cdot-}$ generating oxidase ; NAP_4-NADPH inhibited, however, NADPH oxidase (Figure 2, Traces A and B) suggesting that the NADPH binding site of the enzyme was able to bind NAP_4-NADPH.

The nature of the inhibition caused by NAP_4-NADPH on NADPH oxidation in the presence of cytochrome **c** was assayed at two different fixed

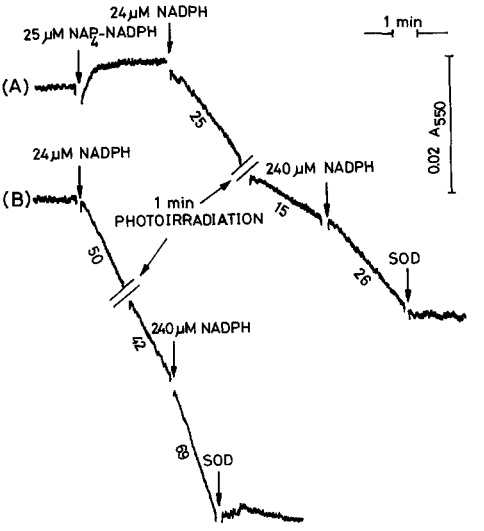

Figure 2. Effect of NAP_4-NADPH on the reduction of cytochrome **c** by NADPH in the presence of the 100000g membrane fraction derived from PMA-activated neutrophils. The membrane fraction (22 μg protein) was suspended in 2 ml of phosphate saline medium supplemented with 100 μM cytochrome **c**. Deoxycholate was added at a low concentration (0.06%) to permeabilise the membranes and favor the oxidation of NADPH by the membrane-bound oxidase. Inhibition of cytochrome **c** reduction by SOD (100 μg) testified that cytochrome **c** reduction was due to $O_2^{\cdot-}$. Where indicated, the suspension in the cuvette was photoirradiated for 1 min with the Xenon lamp XBO 1000WHS placed at 20 cm from the cuvette. The numbers alongside the traces refer to the rates of reduction of cytochrome in nmol/min/mg protein.

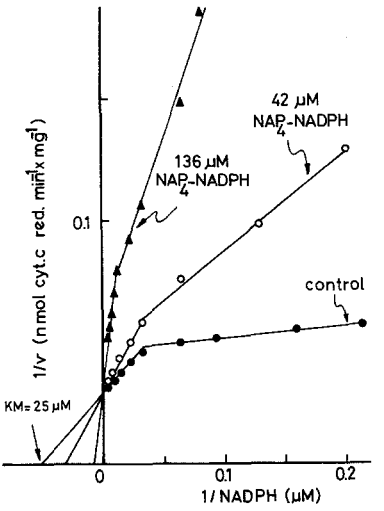

Figure 3. Reciprocal plots of the rate of cytochrome **c** reduction by increasing concentrations of NADPH at two fixed concentrations of NAP_4-NADPH. The experimental conditions were the same as in Figure 2, and NAP_4-NADPH was used at the final concentrations of either 42 µM or 136 µM.

concentrations of NAP_4-NADPH in the absence of photoirradiation. Using the reciprocal plot representation, linear plots were obtained with two distinct regions that can be fitted with straight lines, suggesting the presence of two different NADPH-oxidases in the 100000g membrane fraction (Figure 3). In the control assay, one of the slopes corresponded to a K_M for NADPH of 25 µM and to a SOD-inhibitable reduction of cytochrome **c** ; this slope therefore concerned an $O_2^{\cdot-}$ generating oxidase. The other slope corresponded to a K_M for NADPH of 1 µM and to a SOD-insensitive reduction of cytochrome **c** ; it could be ascribed to a diaphorase activity involved in the direct reduction of cytochrome **c** by NADPH. For concentrations of NADPH higher than 20 µM, at which the $O_2^{\cdot-}$ generating oxidase activity is clearly expressed, a strict competition was demonstrated between NADPH and NAP_4-NADPH.

Photoirradiation of the 100000g membrane fraction with NAP_4-NADPH resulted in an inhibition which was only partially relieved by an excess of NADPH (Figure 2, Traces A and B). The same inhibitory effect was exerted by NAP_4-$NADP^+$. It is therefore concluded that photoirradiation of the 100000g membrane fraction of bovine neutrophils in the presence of NAP_4-$NADP^+$ leads to photoinactivation of the $O_2^{\cdot-}$ generating oxidase.

3. Photolabeling studies with [^{3}H]NAP_4-$NADP^+$ and neutrophil fractions corresponding to different steps of the purification procedure

The 100000g membrane fraction of PMA-activated bovine neutrophils was photoirradiated with 30 µM [^{3}H]NAP_4-$NADP^+$, a concentration of photoprobe

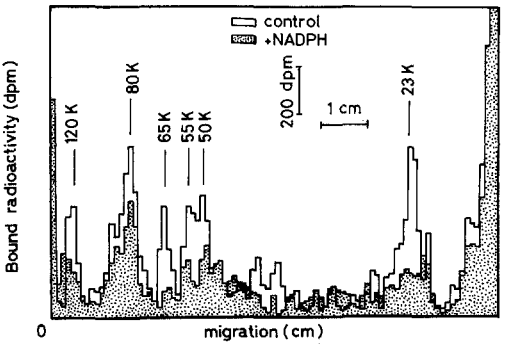

Figure 4. Na Dod sulfate polyacrylamide gel electrophoresis of photolabeled proteins from bovine neutrophils activated by PMA. The 100000g membrane fraction (600 µg protein) recovered from neutrophils was preincubated in the dark for 2 min at 20°C with 30 µM [^{3}H]NAP_4-NADPH and 0.06% deoxycholate in the phosphate-saline medium either with or without prior addition of a large excess of NADPH (1 mM). The suspension was cooled at 0°C and then photoirradiated for 2 min with a 250 W Osram lamp as described in Methods. After addition of 0.5 N perchloric acid at 0°C, the membranes were sedimented by centrifugation and lysed with 1% Na Dod sulfate. The solubilized proteins were subjected to Na Dod sulfate polyacrylamide (10%) gel electrophoresis. The gels were stained with 0.25% Coomasie Blue in acetic acid/methanol/water (1/4/5 v/v) and destained in acetic acid/methanol/water (1/2/7, v/v). After scanning, the gel was sliced. Each slice was digested overnight by 1 ml of 10% H_2O_2 at 65°C. The radioactivity of each digest was measured by liquid scintillation.

sufficient to label efficiently the $O_2^{\cdot-}$ generating oxidase. Photoirradiation was followed by acid precipation of the proteins and solubilization by Na Dod SO_4 ; the solubilized proteins were analyzed by Na Dod SO_4 polyacrylamide gel electrophoresis. The profile of radioactivity in the gel showed a number of peaks corresponding to proteins of Mr 120000, 80000, 65000, 55000, 50000 and 23000 (Figure 4). The effectiveness of photolabeling was influenced greatly by the addition of certain ligands prior to that of NAP_4-$NADP^+$. For example, with an excess of NADPH (1 mM), photolabeling was decreased, indicating the specificity of the photoprobe. However, photolabeling of the 65000 and the 23000 Mr proteins was more decreased than that of the other proteins (Table I and Figure 4). Omission of PMA as activating agent of the respiratory burst in neutrophils resulted in a drastic decrease in the incorporation of photolabel, especially into the proteins of Mr 65000 and 120000. Cibacron Blue and mersalyl, two inhibitors which are effective at micromolar concentrations on the membrane bound oxidase (2, 9) and the purified oxidase (2), also had marked effects on the photolabeling of the 65000 Mr protein (Table I). In summary, among the different photolabeled proteins present in the 100000g membrane fraction of bovine neutrophils, only one protein with a Mr of 65000, was susceptible to a number of ligands tested that are known to interfere with the respiratory burst.

Table 1: Effect of different ligands on photolabeling of neutrophil membrane-bound proteins by $[^3H]NAP_4$-$NADP^+$

Mr (Kd) of photolabeled peaks*	Decrease (%) of the extent of photolabeling depending on the following pretreatments			
	Addition of NADPH	Omission of PMA	Addition of mersalyl	Addition of Cibacron Blue
120	50	77	9	0
80	32	53	34	45
65	77	79	79	62
55	51	36	43	40
50	50	29	37	25
23	68	11	77	48

The 100000g membrane fraction obtained from bovine neutrophils activated by PMA was preincubated in the phosphate saline medium either with 1 mM NADPH, or 50 µM mersalyl or 50 µM Cibacron Blue added prior to the addition of $[^3H]NAP_4$-$NADP^+$ (30 µM). The effect of omission of PMA as activating agent of the neutrophils was also tested. The numbers given in the Table correspond to the percentage decrease in bound $[^3H]NAP_4$-$NADP^+$ under the above mentioned conditions as compared to a control assay. The higest sensitivity to the different pretreatments was found for the 65 KD protein (values underlined).

The radioactivity profiles in Figure 5 were obtained from fractions recovered at different steps of the purification procedure of the $O_2^{\cdot-}$ generating oxidase, namely Triton extraction, DE 52 cellulose chromatography

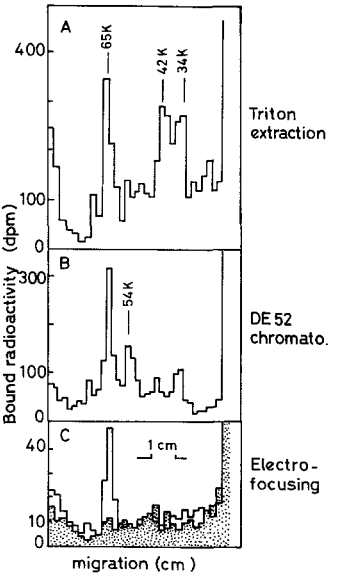

Figure 5. Na Dod sulfate polyacrylamide gel electrophoresis of photolabeled proteins corresponding to different fractions recovered during the course of the purification procedure of the $O_2^{\cdot-}$ generating oxidase. A. Triton X-100 extract of the 100000g membrane fraction (500 µg protein), B. Oxidase-enriched fraction obtained by chromatography of the Triton extract on DE 52 cellulose (250 µg protein), C. Oxidase-enriched fraction obtained by electrofocusing of the DE 52 cellulose fraction (40 µg protein). Dotted bars correspond to photolabeling preceded by preincubation with 1 mM NADPH. Photolabeling of the different fractions with $[^3H]NAP_4$- NADPH was carried out as in Figure 4.

and electrofocusing (2). The first profile was that of a Triton X-100 extract of the 100000g membrane fraction of neutrophils activated by PMA. The second one corresponded to a peak of elution enriched in oxidase activity, obtained by DE 52 cellulose chromatography of the Triton extract. The last profile was that of a fraction with an isoelectric point of 5, recovered by electrofocusing of the DE 52 cellulose fraction. It is noteworthy that some photolabeled proteins present in the 100000g membrane fraction, namely those with Mr of 120000, 80000 and 23000 are not recovered in the Triton extract of this membrane fraction. As 70% of the $O_2^{\cdot-}$ generating oxidase activity is recovered in the Triton extract, this means that the above mentioned proteins are not involved in the $O_2^{\cdot-}$ generating activity. Photolabeling of the 65000 Mr peak predominated in profiles 2 and 3.

DISCUSSION

The present experiments deal with the photolabeling by an arylazido derivative of NADP(H) of an NADPH dependent, $O_2^{\cdot-}$ generating oxidase, located in the plasma membrane of bovine neutrophils. The criteria of affinity and specificity for a photoprobe to be used in photolabeling assays were fulfilled in the present case. In fact, the K_i value for NAP_4-NADPH (40 µM) was similar to the K_M value found for NADPH (25 µM), which indicates that the photoprobe and the natural substrate have similar affinities for the $O_2^{\cdot-}$ generating oxidase. Whereas a number of proteins of the 100000g membrane fraction of bovine neutrophils could be photolabeled by NAP_4-$NADP^+$, the photolabeling of one of these proteins, the 65000 Mr protein, was found to be more sensitive than the others to two powerful inhibitors of the respiratory burst, namely mersalyl and Cibacron Blue. The photolabeling of the 65000 Mr protein was also considerably depressed when activation of bovine neutrophils by PMA was omitted. From these results, it is clear that both the criteria of affinity and specificity apply to the photolabeling of the 65000 Mr protein by NAP_4-$NADP^+$. In addition to these results, a parallelism was found between the oxidase activity enrichment of fractions obtained during the course of the purification procedure of the $O_2^{\cdot-}$ generating oxidase and the photolabeling of a protein of Mr 65000 by [^{3}H]NAP_4-$NADP^+$. These labeling data strongly suggest that among the different membrane proteins of bovine neutrophils able to react with NAP_4-$NADP^+$, the 65000 Mr protein is the only one involved in the respiratory burst.

ACKNOWLEGEMENTS

This work was supported by grants from the "Centre National de la Recherche Scientifique" (CNRS/UA 1130), the "Institut National de la Santé

et de la Recherche Médicale, and the "Faculté de Médecine, Université Scientifique et Médicale de Grenoble". We gratefully acknowledge the excellent secretarial assistance of J. Bournet.

REFERENCES

1. Babior, B.M. (1984) J. Clin. Invest. **73**, 599-601.

2. Doussière, J. and Vignais, P.V. (1985) Biochemistry **24**, 7231-7239.

3. Guillory, R.J. and Jeng, S.J. (1977) Methods Enzymol. **46**, 259-289.

4. Lauquin, G.J.M., Brandolin, G. and Vignais, P.V. (1976) FEBS Lett. **67**, 306-311.

5. Gottikh, B.P., Krayesky, A.A., Tarussova, N.B., Puygin, P.P. and Tsilvich, T.L. (1970) Tetrahedron **26**, 4419-4433.

6. Morel, F., Doussière, J., Stasia, M.J. and Vignais, P.V. (1985) Eur. J. Biochem. **152**, 669-679.

7. Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254.

8. Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. **80**, 575-599.

9. Yamagashi, T. and Kakinuma, K. (1982) Biochem. Biophys. Res. Commun. **104**, 200-206.