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Nitroprusside stimulates the cysteine-specific mono(ADP-ribosylation) of glyceraldehyde-3-phosphate dehydrogenase from human erythrocytes

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In human erythrocyte membranes incubated with [adenylate-^{32}P]NAD the 36 kDa protein is predominantly labeled. The labeling is greatly stimulated by nitroprusside in the presence of dithiothreitol. We have purified the 36 kDa protein and identified this modification as cysteine-specific mono(ADP-ribosylation) because: (i) labeling occurred only when [^{32}P]NAD was replaced by adenine [U-^{14}C]NAD, but not by [carbonyl-^{14}C]NAD; (ii) treatment of the prelabeled protein with snake venom phosphodiesterase led to releasing 5′-[^{32}P]AMP; (iii) the bond between the protein and the nucleotide was hydrolyzed by $HgCl_2$, but was resistant to hydroxylamine. The 36 kDa protein reacted on Western blots with two different monoclonal antibodies (MAbs) against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and was immunoprecipitated by both MAbs.

Glyceraldehyde-3-phosphate dehydrogenase; Human erythrocyte; ADP-ribosylation; Sodium nitroprusside

1. INTRODUCTION

ADP-ribosylation is an important mechanism in the post-translational modification of cellular proteins. Mono(ADP-ribosylation) is involved in the effects of cholera [1] and pertussis toxins [2]. Endogenous ADP-ribosyl transferases, identified in various eukaryotic cells, may use the same substrates as toxins do. Arginine-specific [3] and cysteine-specific [4] enzymes share common substrates with cholera and pertussis toxins, respectively. Other endogenous substrates of 36 and 39 kDa are not targets of bacterial toxins [5]. The cysteine residues of unknown membrane proteins are bound to ADP-ribose in vivo [6]. The 39 kDa protein of platelet cytosol is ADP-ribosylated only endogenously. This reaction is stimulated by NO-generating substances [7].

Recently, we found that human erythrocyte membrane proteins of 41 kDa and 37 kDa could be endogenously ADP-ribosylated [8]. In the present study we have investigated the stimulatory effect of nitroprusside on the cysteine-specific ADP-ribosylation of the 36 kDa protein. We purified the 36 kDa protein (the apparent molecular mass of the labeled protein is 37 kDa), identified as glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.2.12) (GAPDH).

2. MATERIALS AND METHODS

[adenylate-^{32}P]NAD (10–20 Ci/mmol) was synthesized from [α-^{32}P]ATP (Amersham) as described [9]. [Carbonyl-^{14}C]NAD (59.2 mCi/mmol) and adenine [U-^{14}C]NAD (296 mCi/mmol) were from Amersham. Reagents for electrophoresis and blotting were obtained from Bio-Rad, a molecular mass marker protein kit was from Sigma, nucleotides were purchased from Boehringer Mannheim. All other chemicals were of analytical grade. Protein was determined by staining with Amido black [10]. Preparations of GAPDH, purified from yeast and *E. coli* were the gift of Dr. E.V. Kuzminskaya and Dr. N.A. Khoroshilova, respectively (Institute of A.N. Belozerski, Moscow State University).

2.1. *Purification of GAPDH from human erythrocyte membranes*

Erythrocyte membranes were prepared from fresh human blood by the method of Dodge et al. [11] with slight modifications. Briefly, cells were lysed with 5 vols. of a hypotonic solution, containing 5 mM sodium phosphate (pH 8.0), 1 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, the pellet was lysed three times with the same solution and membranes (15 mg of protein/ml) were used the same day. 20 ml of membranes were washed two times with 100 ml of 20 mM HEPES-NaOH (pH 7.8), extracted in 15 ml of 20 mM HEPES-NaOH (pH 7.8), containing 0.2 M NaCl and centrifuged at 100,000 × g for 1 h. The supernatant was concentrated using an Amicon PM-10 membrane to 0.7 ml and applied on an Sephacryl S-200 (Pharmacia) column (1.6 × 26 cm), equilibrated with 20 mM HEPES-NaOH (pH 7.8), containing 50 mM NaCl. The 36 kDa protein was eluted with the same buffer and fractions with an apparent molecular mass of 70–75 kDa were pooled (data not shown).

2.2. *ADP-ribosyl transferase assay*

The incubation mixture for ADP-ribosylation of membranes at a final volume of 40 μl contained 50 mM Tris-HCl (pH 7.8), 10 mM DTT, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM thymidine, 1 mM NADP, 1–2 μCi [^{32}P]NAD (1 μM), 1 mM sodium nitroprusside and 40 μg of membrane protein. Incubation mixture for ADP-ribosylation of the purified 36 kDa protein (2 μg/sample) did not contain thymidine and NADP, concentration of sodium nitroprusside was reduced to 0.1 mM. Samples were incubated at 37°C for 1 h, the reaction was stopped by the addition of 10 μl of the denaturating mixture [8]. Samples were boiled, and proteins were resolved by 11% polyacrylamide gels by the method of Laemmli [12]. Dry gels were exposed to a standard X-ray

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film for 12–16 h (^{32}P) or 2–3 weeks (^{14}C). The bands of interest were excised from the gel, dissolved in 30% H_2O_2, 1% NH_4OH and counted by dioxane scintillation. Bonds of proteins and ADP-ribose were cleaved as described elsewhere [13].

2.3. *HPLC separation of nucleotides*

Western blotting was performed using a BioTecMed ApS (Denmark) apparatus for semi-dry electrophoretic transfer. Nitrocellulose filters were treated with a solution, containing 1 mM Tris-HCl (pH 7.5), 1% bovine serum albumin, 0.1% Tween 20; protein bands of 36–37 kDa were excised, and filter sheets were treated by snake venom phosphodiesterase (50 μg/ml) at 30°C for 1 h. Samples were boiled, lyophylized and dissolved in 100 μl of 25 mM potassium phosphate (pH 3.5), containing AMP, ADP, ADP-ribose and NAD, at 0.1 mM concentration each. The solution was applied on the Ultrasphere-ODS column, 4.6 × 250 mm (ALTEX, USA) equilibrated with 25 mM potassium phosphate (pH 3.5). Nucleotides were eluted with the same buffer at a flow rate of 0.5 ml/min. Nucleotides were also resolved by thin-layer chromatography on either cellulose or PEI-cellulose plates (Merck) [14].

2.4. *Immunoblotting*

Mouse monoclonal antibodies (MAbs) C5 and G7 raised against rabbit muscle GAPDH were kindly provided by Dr. A.G. Katrukha (Moscow State University, Laboratory of Enzyme Chemistry). MAbs were affinity-purified from ascitic fluid by chromatography on GAPDH-Sepharose prepared by coupling the rabbit muscle enzyme to CH-activated Sepharose-4B (Pharmacia). Antibody isotype was determined using mouse hybridoma sub-isotyping kit (Calbiochem). The proteins were transferred to nitrocellulose, blots were incubated in the solution containing 10 mM Tris-HCl, pH 7.8, 1% bovine serum albumin, 0.1% Tween 20 and 10 μg of affinity-purified MAbs/ml, and GAPDH-corresponding bands were stained by the immunoperoxidase method using goat rabbit anti-mouse peroxidase-conjugated antibodies (Calbiochem) as described [15].

2.5. *Immunoprecipitation*

The ADP-ribosylation reaction was stopped by the addition of 1 mM unlabeled NAD, 1 ml of the immunoprecipitation buffer, containing 1% Triton X-100, 0.1% SDS, 0.4 M NaCl, 10 mM EDTA, 0.1 M potassium phosphate, 50 mM HEPES-NaOH, pH 7.2, and 40 μg of affinity-purified MAbs were added to each sample and incubated overnight at 4°C. After addition of 30 μl of Protein A-Sepharose (Pharmacia, 20 μl of packed gel), samples were incubated for 1 h at 30°C, centrifuged, the pellet was washed four times with the immunoprecipitation solution. Denaturation mixture was added, samples were boiled and applied to gel. When the G7 mAb was used, Protein A-Sepharose was preincubated with an excess of rabbit anti-mouse antibodies (Calbiochem).

3. RESULTS AND DISCUSSION

Sodium nitroprusside is a hypotensive drug. It is metabolysed in blood vessels and nitric oxide generated is thought to cause the relaxation of smooth muscle cells [16]. Natural mechanisms involving NO as a plausible cellular regulator are proposed [17]. Nitric oxide stimulates the endogenous ADP-ribosylation of the 39 kDa protein in many cell types [8]. Our results show the same effect for the ADP-ribosylation of the 36 kDa protein of human erythrocytes.

Human erythrocyte membranes were incubated with [adenylate-^{32}P]NAD. The predominant labeling of the 36 kDa protein was detected. Sodium nitroprusside greatly (approximately 20-fold) stimulated the modification (Fig. 1). The 36 kDa protein was extracted by treatment of membranes with 0.2 M NaCl and purified to apparent homogeneity by subsequent concentration and gel filtration. The polypeptide obtained was still modified by [^{32}P]NAD and the reaction was stimulated by sodium nitroprusside to the same extent (Fig. 1). Sodium nitroprusside exhibited stimulatory effect only in the presence of dithiothreitol (Fig. 1). This is consistent with the nitroprusside action on guanylate cyclase [18].

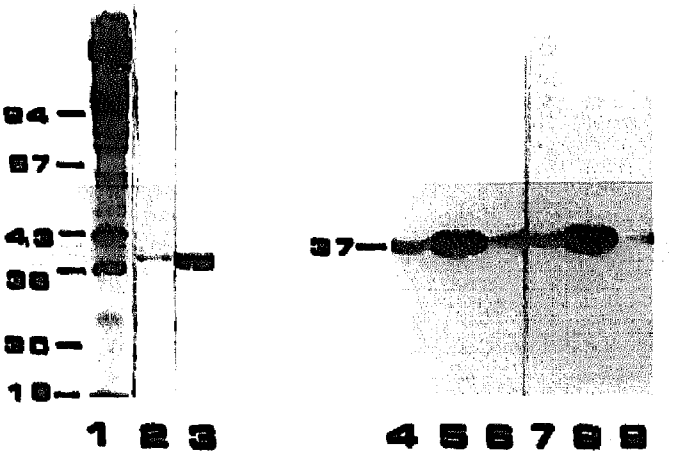

Fig. 1. ADP-ribosylation of the 36 kDa protein of human erythrocytes. Membranes (40 μg of protein; lanes 1, 4–6) and the purified 36 kDa protein (2 μg; lanes 2, 7–9 and 20 μg; lane 3) were treated with [^{32}P]NAD as described in section 2.2. Gels were stained with Coomassie R-250 (lanes 1–3) and autoradiographed (lanes 4–9). Incubation was carried out in the absence of nitroprusside (lanes 4 and 7), with nitroprusside (lanes 5 and 8), with nitroprusside but in the absence of dithiothreitol (lanes 6 and 9). Numbers on the left indicate molecular mass in kDa.

Sodium nitroprusside is presumed to stimulate the cysteine-specific mono (ADP-ribosylation) of the 36 kDa protein for the following reasons.

First, [^{32}P]NAD was replaced either by adenine

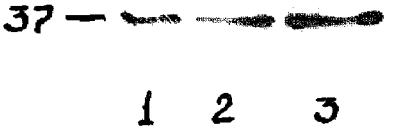

Fig. 2. Specific removal of [^{32}P]ADP-ribose from the 36 kDa protein. The purified 36 kDa protein (20 μg/sample) was labeled with [^{32}P]NAD in the presence of sodium nitroprusside. The reaction was stopped by the addition of unlabeled 1 mM NAD and 1% bovine serum albumin, protein was pelleted by cold 5% trichloroacetic acid, the pellet was dissolved in 0.1 M HEPES-NaOH (pH 7.8) and incubated at 37°C without additions for 3 h (lane 1), with 0.5 M hydroxylamine (pH 7.5) for 30 min (lane 2) and 3 h (lane 3), with 1 mM $HgCl_2$ for 30 min (lane 4), with snake venom phosphodiesterase (50 μg/ml) and 1 mM $MgCl_2$ for 30 min (lane 5). The denaturing mixture was added, samples were boiled and electrophoresed. The autoradiograms are presented.

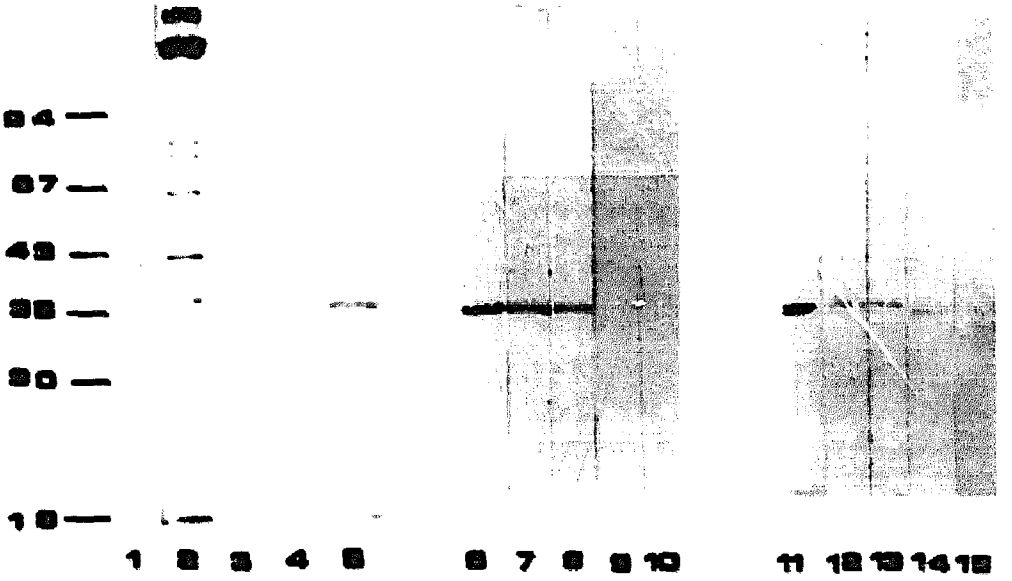

Fig. 3. Immunoblotting of GAPDH. Proteins were electrophoresed on 11% polyacrylamide gels and stained by Coomassie (lanes 1–5) or transferred to nitrocellulose filters, treated by MAbs C5 (lanes 6–10) or G7 (11–15), as described in section 2.4. 1, 6, 11: 1 μg of rabbit muscle GAPDH; 2, 7, 12: 10 μg of human erythrocyte membrane proteins; 3, 8, 13: 1 μg of the purified 36 kDa protein; 4, 9, 14: 1 μg of yeast GAPDH; 5, 10, 15: 1 μg of GAPDH from *E. coli*. Numbers on the left indicate molecular mass in kDa.

[U-^{14}C]NAD or [carbonyl-^{14}C]NAD at 5 mM concentration each and specific activity 40 mCi/mmol with or without nitroprusside. The isotope was incorporated only when adenine [U-^{14}C]NAD was used as a substrate, but no labeling occurred with [carbonyl-^{14}C]NAD. Thus, the nicotinamide moiety of NAD is not bound to the modified polypeptide. Incorporation of adenine-[U-^{14}C]ADP-ribose was 8 mmol/mol of polypeptide and was maximal for this preparation, because preliminary results show that conditions described in section 2.2. are optimal for the reaction.

Second, prelabeled 36 kDa protein was blotted to nitrocellulose, the bound nucleotide was hydrolyzed by snake venom phosphodiesterase and identified as 5′-[^{32}P]AMP (Table I).

Third, the bond between the 36 kDa protein and ADP-ribose was cleaved by snake venom phosphodiesterase and $HgCl_2$, but not by hydroxylamine (Fig. 2). It is known that bonds between protein and ADP-ribose are hydrolyzed by several specific reagents. Cysteine-ADP-ribose is cleaved by $HgCl_2$, arginine-ADP-ribose by hydroxylamine and both by snake venom phosphodiesterase [13].

Such characteristics of the 36 kDa protein as well as its apparent molecular mass, relative amount in

Table I

Chromatographic analysis of nucleotides, released after snake venom phosphodiesterase treatment of the prelabeled 36 kDa protein. Protein was treated with phosphodiesterase and nucleotides were analysed as described in section 2.3.

Method of resolution	Relative amount of ^{32}P-containing nucleotides (% of total)			
	AMP	ADP	ADP-ribose	NAD
HPLC	93	3	2	2
TLC on PEI-cellulose	99	-	1	-
TLC on cellulose	95	-	5	-

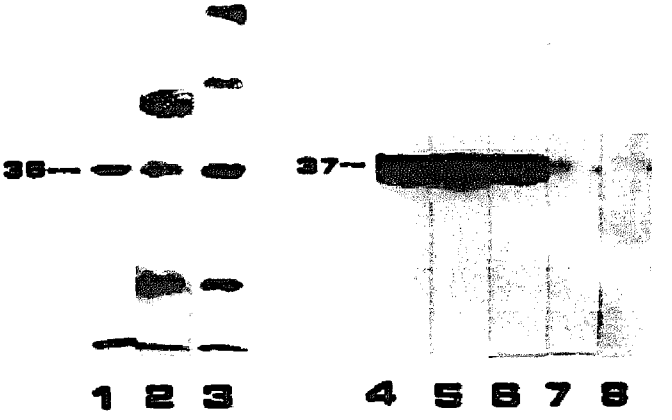

Fig. 4. Immunoprecipitation of GAPDH. Immunoprecipitation was performed as described in section 2.5. Gels were stained with Coomassie blue (lanes 1–3) and autoradiographed (lanes 4–8). 1, 4: control sample, not subjected to immunoprecipitation; 2, 5: immunoprecipitation of ADP-ribosylated protein by mAb C5; 3, 6: immunoprecipitation of ADP-ribosylated protein by mAb G7; immunoprecipitation was carried out in the presence of 50 μg of rabbit muscle GAPDH with MAbs C5 (7) or G7 (8). Numbers on the left indicate molecular mass in kDa.

membranes and ability to be extracted by solutions with increasing ionic strength, made it possible to suggest this protein to be GAPDH. We used two mouse MAbs raised against rabbit muscle GAPDH, and determined by immunoblotting that both MAbs, C5 and G7 (IgG2b and IgM, respectively) recognized only the 36 kDa protein in human erythrocyte membranes and the purified 36 kDa protein (Fig. 3). Both MAbs reacted with rabbit muscle GAPDH, only MAb G7 reacted with GAPDH from yeast, and none of the MAbs reacted with the enzyme from *E. coli* (Fig. 3). Hence, these MAbs are probably reacting with different sites of the GAPDH molecule. Moreover, both MAbs were able to immunoprecipitate the ADP-ribosylated protein from the purified 36 kDa protein preparation, and this reaction was inhibited in the presence of excess of rabbit muscle GAPDH (Fig. 4). Tanaka et al. described a non-enzymic ADP-ribosylation of the 36 kDa protein (appeared to be GAPDH) in the cytosol of rat skeletal muscle, although the direct identification of endogenous 36 kDa protein as GAPDH was not shown [5]. In the present paper we obtained a strong evidence that the 36 kDa substrate of nitroprusside-stimulated ADP-ribosylation in human erythrocytes is GAPDH (Figs. 3 and 4).

The functional role of ADP-ribosylation of GAPDH is unknown. The low level of label incorporation (8 mmol/mol of GAPDH monomer) suggests that such a modification may affect the enzyme activity in the entire cell insignificantly. It is possible that the cysteine residue had been already endogenously modified, thus decreasing the observed value of label incorporation. It is important to determine whether the contaminating proteins, such as cysteine-specific ADP-ribosyl transferase might be a target of NO, although we have found no contaminations in our preparation of GAPDH (Fig. 1). More likely, nitric oxide exerts its action on GAPDH directly.

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