**NAD⁺-dependent post-translational modification of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase**

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**Summary.** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional housekeeping protein reported to be a target of several covalent modifications in many organisms. In a previous study, enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains were shown to secrete GAPDH and the protein to bind human plasminogen and fibrinogen. Here we report that GAPDH of these pathogens is ADP-ribosylated either in the cytoplasm or in the extracellular medium. GAPDH catalyzes its own modification, which involves Cys-149 at the active site. ADP-ribosylation of extracellular GAPDH may play an important role in the host-pathogen interaction, as also proposed in other pathogens. [Int Microbiol 2009; 12(3):187-192]

**Keywords:** *Escherichia coli* · glyceraldehyde-3-phosphate dehydrogenase (GAPDH) · secreted proteins · ADP-ribosylation · covalent modification of proteins

**Introduction**

Several cytoplasmic housekeeping enzymes with no detectable secretion and retention signal are present on the surface of microbial pathogens, where they perform functions related to the adhesion and/or virulence of the pathogen [13]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is among these secreted proteins that interact with different host components [14,19]. In gram-negative bacteria, the extracellular location of GAPDH in enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains was reported by our group [5]. This protein is secreted into the medium in a soluble and active form and is also found on the cell surface of these pathogens. *E. coli* GAPDH binds human plasminogen and fibrinogen and remains associated with colon epithelial Caco-2 cells after the adhesion of EHEC or EPEC [5].

In many organisms, GAPDH can be a target of several covalent modifications including glutathionylation, S-nitrosylation, binding of nitrated fatty acids, phosphorylation, and ADP-ribosylation [2-4,16]. Most of these modifications are linked to oxidative stress responses and may be crucial for proper adaptation to conditions of oxygen stress [3,16,17]. ADP-ribosylation is a reversible, post-translational, covalent modification of proteins in which the ADP-ribose moiety of NAD⁺ is enzymatically transferred to specific amino acid residues (His, Arg, or Cys) of the target protein, with the subsequent release of nicotinamide [7,10,20]. This modification
regulates protein function and affects many biological processes. For instance, in *Entamoeba histolytica* or group A streptococci ADP-ribosylation of secreted GAPDH has been implicated in the interaction of these pathogens with the host [1,15]. Here, we provide the first report of covalent modification of GAPDH by ADP-ribosylation in gram-negative bacteria.

**Materials and methods**

**Growth conditions.** Bacterial cells were routinely grown at 37°C in Luria-Bertani (LB) broth. The gapA mutant strain W3CG was grown in minimal medium supplemented with malate and glycerol as the carbon source [6]. Where indicated, cells were grown without shaking at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) in a CO2 incubator. Growth was monitored by measuring the optical density at 600 nm (OD600). When required, tetracycline (12.5 µg/ml) or ampicillin (100 µg/ml) was added to the medium.

**Preparation of cell extracts and isolation of secreted proteins in culture medium.** Overnight cultures in LB were diluted 1:50 in the indicated culture media and incubated without shaking at 37°C in a 5% CO2 atmosphere. The bacteria were collected by centrifugation (5000 xg, 10 min, 4°C) and resuspended in appropriate buffer. After sonication on ice, cell debris was removed by centrifugation at 16,000 xg for 30 min at 4°C. The protein concentration of the cell extract was determined by the method of Lowry [11]. Secreted proteins were isolated by filtering the culture supernatant through a 0.22-µm pore size filter (Millipore), and the proteins in the filtrate were precipitated by incubation on ice for at least 1 h with 10% trichloroacetic acid (TCA). The protein pellet was washed in 90% (v/v) ice-cold acetone, air-dried, and suspended in loading buffer before being resolved by SDS-PAGE [8]. For ADP-ribosylation assays, the secreted proteins were concentrated from 100 ml of the filtrated supernatant by 95% ammonium sulfate precipitation. The protein pellets were resuspended in 0.5 ml of 100 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol (DTT) and then dialyzed overnight against the same buffer.

**Purification of recombinant GAPDH.** Recombinant GAPDH was expressed and purified using the glutathione-S-transferase (GST) gene fusion system with recognition sites for factor Xa cleavage, as described in detail previously [5].

**ADP-ribosylation assays.** The ADP-ribosylation reaction mixtures (100 µl) contained: 100 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM ATP, 2 mM MgCl2, 80 µM E-64, 2 mM NAD+ plus 2 µCi [32P]NAD+ (51 mCi/mmol), and protein sample (75 µg secreted proteins or 200 µg cell extracts) as the source of both enzyme and substrate(s). When indicated, reactions were done with 10 µg of purified recombinant GAPDH. In some experiments, 100 µM non-radioactive NAD+ or 100 µM non-radioactive ADP-ribose was added as putative competitor. The effect of HgCl2 or hydroxylamine (pH 7.4) on NAD+-dependent modification was determined by incorporating these reagents at concentrations up to 5 mM into the reaction buffer prior to the addition of [32P]NAD+. ADP-ribosylation reactions were incubated for 1 h at 37°C followed by the addition of 25 µl of 4× SDS-loading buffer to stop the reaction. The samples were heated to 70°C for 20 min and subjected to 12% SDS-PAGE [8]. The gel was dried and autoradiographed at ~80°C.

**Western blot.** Western blot analysis using specific antibodies against *E. coli* GAPDH was carried out as described previously [5].

**Two-dimensional gel electrophoresis.** 2D gel electrophoresis was performed using the Protean IEF-Cell (Bio-Rad). Appropriate volumes of protein samples were diluted in 125 µl of rehydration buffer (9 M urea, 4% CHAPS, 50 mM DTT, 0.5% immobilized pH gradient buffer, and traces of bromophenol blue). Isoelectric focusing was carried out in immobilized pH 5–8 gradient strips (BioRad). Second-dimension SDS-PAGE was done on 12.5% acrylamide gels.

**Mass spectrometry of proteins.** Following SDS-PAGE [8], the gels were stained and the protein bands of interest excised and digested with trypsin (Sequencing grade modified, Promega) in the automatic Investigator ProGest robot of Genomic Solutions. Peptides released from the gel were analyzed by combined liquid chromatography/tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF) (CapLC, Micromass-Waters) at the Scientific-Technical Services of the University of Barcelona. Data were generated in PKL file format, which were submitted for database searching in the MASCOT server.

**DNA manipulation and site-directed mutagenesis.** DNA manipulations in vitro were carried out according to standard protocols [18]. Site-directed mutagenesis was done using the QuickChange PCR-based mutagenesis procedure (Stratagene) with pGEX-GapA [5] as the template. The primers used to construct the GAPDH C154A mutant were gapdh-mut1-fw (5’-GGTTCACAGCGTTTCCGCTGCTCCTCACAAAATCGCTG-3’) and gapdh-mut2-rv (5’-CAAGGCGATGTTGTTAGGGGAAAGCTTGGGAAAAC-3’). To construct C153A, the mutant primers were gapdh-mut2-fw (5’-CCTGCAACACACAGCTGCTCCGCCTGCC3’) and gapdh-mut2-rv (5’-GCCAGGGAGCGAGCGGTGGTGACGG-3’), and for C288A mutant, gapdh-mut3-fw (5’-GATTTCACACGCGGAAATGTCCTCCGGTGTTGGG-3’) and gapdh-mut3-rv (5’-CGAACACGGAATGACCACTTGGCACCCTGGGATACAC-3’). The mutated codons are underlined. The appropriate substitutions and the absence of mutations were confirmed by sequencing the inserts in both directions.

**Results and Discussion**

**Identification of GAPDH as an ADP-ribosylated protein in *Escherichia coli*.** To identify secreted proteins that could be ADP-ribosylated in EHEC or EPEC strains, cell-free supernatants of EHEC strain 86-24h11 or EPEC strain E2348/69 [5] grown in DMEM were concentrated by ammonium sulfate precipitation. These samples were incubated under ADP-ribosylation conditions and processed as described in Materials and methods. Two protein bands, of 35 and 72 kDa, were radio-labeled in these conditions (Fig. 1A, lanes 1 and 4). The presence of non-radioactive NAD+ inhibited the labeling of both proteins (Fig. 1A, lanes 2 and 5) whereas non-radioactive ADP-ribose did not modify the ADP-ribosylation signal (Fig. 1A, lanes 3 and 6). These results indicate that the covalent modification of both proteins depends on NAD+.

The same 35 and 72 kDa protein bands were visualized when the ADP-ribosylation reactions were carried out with cell extracts of EHEC, indicating that these proteins are also modified in the intracellular medium (Fig. 1B). The fact that these results hold true for cell extracts of the non-pathogenic
strains ECL1 [9] and EcoR26 [12] suggested that this modification affects proteins expressed in all *E. coli* strains (Fig. 1B). In all experiments done with cell extracts, the labeled proteins appeared as fainter bands, which can be attributed to the presence of intracellular ADP-ribosylhydrolases, as described in other prokaryotes [15]. Likewise, when EHEC-secreted protein samples were incubated at 37°C with the corresponding cytoplasmic fraction in [32P]ADP-ribosylation reaction mixtures, the radioactivity incorporated into the 35 and 72 kDa proteins was significantly reduced (not shown). This suggests that in vivo NAD+-dependent modification of these proteins can be blunted by intracellular factors.

The finding that the labeled 35 kDa protein was absent in cell extracts of the *gapA* mutant strain W3CG [6] suggested that GAPDH was one of the protein targets of post-translational modification by NAD+ (Fig. 1B). This was further supported by the results of Western blot analysis of ADP-ribosylation reactions carried out with cell-free supernatants of EHEC. A 35-kDa protein band coincident with that labeled in the presence of [32P]NAD+ was detected with specific antibodies against GAPDH (lane 2). To confirm the identity of the 35-kDa protein as GAPDH, non-radioactive ADP-ribosylation samples were electrophoresed in parallel and the protein band coincident with the radioactive signal was excised from a silver-stained duplicate gel (Fig. 1C, lane 3), digested with trypsin, and analyzed by mass spectrometry. This allowed the identification of several peptides belonging all to *E. coli* GAPDH (Fig. 1D).
To examine whether *E. coli* GAPDH is able to promote its own modification, as described for the GAPDH of other organisms [15], recombinant GAPDH was incubated with [³²P]NAD⁺ under ADP-ribosylation conditions. As shown in Fig. 2A, the purified enzyme showed radioactive labeling, which clearly diminished in the presence of non-radioactive NAD⁺. In a previous study, the results of two-dimensional (2D) gel electrophoresis analysis of recombinant GAPDH showed that there are multiple forms of GAPDH which differ in their pI [5]. Here, a similar 2D-analysis was done with GAPDH incubated under ADP-ribosylation conditions. The gels were dried and either autoradiographed at –80°C or processed for anti-GAPDH Western blotting. Parallel gels were silver-stained to visualize total protein. Results of this 2D analysis showed that the ADP-ribosylated form corresponded to a minor spot displacing the lowest pI (Fig. 2B).

**Identification of Cys-149 as the amino acid acceptor of the NAD⁺-dependent modification.**

In an attempt to identify which residue was modified by NAD⁺, ADP-ribosylation reactions were carried out in the presence of increasing concentrations (0–5 mM) of hydroxylamine (which inhibits Arg-specific ADP-ribosylation), or HgCl₂ (which inhibits Cys-specific ADP-ribosylation) [15]. The presence of hydroxylamine did not affect the radioactivity incorporated into recombinant GAPDH, whereas HgCl₂ inhibited ³²P-incorporation in a dose-dependent manner (Fig. 3A). The same result was obtained when the secreted protein fraction was used as a substrate in the ADP-ribosylation reaction (not shown). These results suggest that the NAD⁺-dependent modification of *E. coli* GAPDH occurred through a thiol linkage to a Cys residue. To test this hypothesis, competitive inhibition of GAPDH ADP-ribosylation was carried out in the presence of an excess of free L-Cys, L-Arg or L-His, which are ADP-ribose acceptors. Consistently, GAPDH labeling in the presence of [³²P]NAD⁺ was only inhibited by L-Cys and this inhibition was dose-dependent (Fig. 3B). Inhibition by L-Cys was also observed with the secreted protein (not shown).

There are three Cys residues in *E. coli* GAPDH, Cys-149, Cys-153, and Cys-288. To identify which of these residues was the target of the NAD⁺-dependent modification, each Cys residue was changed to Ala. The mutated proteins were expressed and purified as described for the wild-type protein [5] and then incubated with [³²P]NAD⁺ under ADP-ribosylation conditions. Mutations C153A or C288A did not impair radioactive incorporation into the protein, whereas mutation C149A abolished NAD⁺-dependent labeling (Fig. 3C). These results clearly pointed to the active-site Cys-149 as the target residue of this modification.
Since ADP-ribosylation is involved in signal transduction events, our results point to additional roles for extracellular GAPDH in the interaction of EHEC and EPEC strains with the host, as proposed for other pathogens [1,15]. Moreover, since this reversible NAD+-dependent modification also takes place in the cytoplasm of pathogenic and non-pathogenic E. coli strains, GAPDH may also play a regulatory role within E. coli cells under physiological conditions.

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References


