Protein interaction studies point to new functions for *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase

Elaine Ferreiraa, Rosa Giméneza, Laura Aguileraa, Karla Guzmán, Juan Aguilara, Josefa Badiaa, Laura Baldomàa*

aDepartament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Institut de Biomedicina de la Universitat de Barcelona (IBUB), Universitat de Barcelona, Av. Diagonal, 643. E-08028 Barcelona, Spain,

Elaine Ferreira: elainefmelo@gmail.com
Rosa Giménez: rgimenez@ub.edu
Laura Aguilera: laguilera@ub.edu
Karla Guzmán: guz_krl@hotmail.com
Juan Aguilar: juanaguilar@ub.edu
Josefa Badia: josefabadia@ub.edu
Laura Baldomà: lbaldoma@ub.edu “Correspondence and reprints”
Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is considered a multifunctional protein with defined functions in numerous mammalian cellular processes. GAPDH functional diversity depends on various factors such as covalent modifications, subcellular localization, oligomeric state and intracellular concentration of substrates or ligands, as well as protein-protein interactions. In bacteria, alternative GAPDH functions have been associated with its extracellular location in pathogens or probiotics. In this study, new intracellular functions of *E. coli* GAPDH were investigated following a proteomic approach aimed at identifying interacting partners using *in vivo* formaldehyde cross-linking followed by mass spectrometry. The identified proteins were involved in metabolic processes, protein synthesis and folding or DNA repair. Some interacting proteins were also identified in immunopurification experiments in the absence of cross-linking. Pull-down experiments and overlay immunoblotting were performed to further characterize the interaction with phosphoglycolate phosphatase (Gph). This enzyme is involved in the metabolism of 2-phosphoglycolate formed in the DNA repair of 3’-phosphoglycolate ends generated by bleomycin damage. We show that interaction between Gph and GAPDH increases in cells challenged with bleomycin, suggesting involvement of GAPDH in cellular processes linked to DNA repair mechanisms.

Keywords: moonlighting proteins; glyceraldehyde-3-phosphate dehydrogenase; protein-protein interactions; phosphoglycolate phosphatase; DNA repair; *Escherichia coli*
Abbreviations: CAA, casein acid hydrolysate CAA; CFUs, colony forming units; FA, formaldehyde; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gph, phosphoglycolate phosphatase; GST, glutathione-S-transferase; MS, mass spectrometry; NBRP, National BioResource Project; PVDF, polyvinylidene difluoride.
1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme in the glycolytic pathway, is not merely a classical metabolic protein, but is also considered a multifunctional protein with defined functions in numerous mammalian cellular processes (reviewed in Sirover, 1999, 2005, 2011). This protein is involved in transcriptional and post-transcriptional gene regulation, chromatin structure, intracellular membrane trafficking, DNA replication and DNA repair processes (Sirover, 2011). Each new function requires GAPDH association into specific protein complexes. The functional diversity of GAPDH depends on various factors, such as covalent modifications, subcellular localization, oligomeric state, or intracellular concentration of substrates (Jeffery, 2004; Sirover, 2005, 2011). In this context, it is important to consider the great diversity of post-translational modifications described for GAPDH, which may underpin its multifunctional activity (Sirover, 2011). Many of these modifications are associated with various types of stress (Cabiscoł and Ros, 2006; Colussi et al., 2000; Eaton et al., 2002).

In bacteria, alternative GAPDH functions have been associated with its extracellular location in pathogens. GAPDH is one of the housekeeping proteins that are secreted and exposed on the bacterial surface, enabling the pathogens to colonize and persist in the host (Pancholi and Chhatwal, 2003). Several reports on different gram-positive pathogens have demonstrated the ability of extracellular GAPDH to interact with various host components such as transferrin, plasminogen, extracellular matrix components or the urokinase plasminogen activator receptor (Jin et al., 2005; Schaumburg et al., 2004; Seifert et al., 2003). In the case of the gram-negative pathogens enteropathogenic E. coli (EPEC) and enterohaemorrhagic E. coli (EHEC), our group showed that secreted GAPDH is able to
interact with human plasminogen and fibrinogen, and remains associated with Caco-2 cells upon infection (Egea et al., 2007). Extracellular GAPDH can also act as a target for oxidation and therefore may protect bacteria against host oxidative response (Aguilera et al., 2009). In recent years, GAPDH has been identified in the secretome of probiotic strains (Aguilera et al., 2012; Sanchez et al., 2009a, 2009b). Extracellular GAPDH may confer these strains an advantage in gut colonization with respect to other components of the intestinal microbiota. Besides the role of secreted GAPDH in host colonization processes, no studies of new GAPDH intracellular functions have been described so far in bacteria. In the area of systems biology, the identification of protein-protein interactions within cells is of prime importance to understand biological processes at the molecular level. Several groups have performed large-scale analyses using *E. coli* to identify interactions between proteins (Arifuzzaman et al., 2006; Butland et al., 2005). These studies, based on tagged protein baits that allow affinity purification of the interacting proteins, have shown that GAPDH interacts with a range of proteins, including metabolic enzymes and proteins involved in transcription or protein synthesis, suggesting the potential role of *E. coli* GAPDH in some cellular processes similar to those described in humans.

When using these affinity-based methods, transient interactions or weakly binding proteins can be lost during washing procedures. As an alternative, *in vivo* chemical cross-linking experiments can be performed to covalently fix interaction partners in living cells, thus preventing the loss of specific components of a protein complex prior to purification (Sinz, 2010). Although formaldehyde is widely used as a cross-linker in chromatin immunoprecipitation experiments, the number of studies which have used this reagent in combination with mass spectrometry (MS) to investigate protein-protein interaction is
 limited (Sinz, 2010). Examples of this approach are the identification of new proteins interacting with a constitutive active form of M-Ras (Vasilescu et al., 2004) or the identification of glutamate dehydrogenase as a protein that interacts with the regulator GltC in *Bacillus subtilis* (Herberg et al., 2007).

To explore new GAPDH functions in *E. coli*, in this study we conducted *in vivo* formaldehyde cross-linking experiments followed by MS to identify proteins interacting with V5-tagged GAPDH in *E. coli*. We also characterized interaction with phosphoglycolate phosphatase (Gph), an enzyme involved in the metabolism of 2-phosphoglycolate, a compound formed in the DNA repair of 3’-phosphoglycolate ends generated by bleomycin damage (Pellicer et al., 2003).

2. Materials and methods

2.1. Bacterial strains and growth conditions

The genotypes and sources of the *E. coli* strains used in this study are as follows. Strain MC4100 (F− *araD* Δ(*argF-lac*) *rpsL*(Strr) *relA* *fihD* *deoC* *ptsF* *rbs*) (Casadaban, 1976) was the source of chromosomal DNA used as a template for PCR amplification of gene *gapA*. The mutant strain JA210 (*gph::Kan*) was derived from MC4100 (Pellicer et al., 2003). Strain TOP10 (F− *∆mrcA* (mrr-ḥsdRMS- mcrBC) φ80lacZΔM15 ΔlacX74 *recA1* *araD139* Δ(ara-leu)7697 *galU* *galK* *rpsL* (Strr) *endA1* *nupG*) was used to express recombinant GAPDH-V5 (Invitrogen). Strain BL21(DE3) (F− *ompT hsd(γ− mB−) gal*) was used as a host to express GST-tagged proteins (Amershan Pharmacia). Strain AG1 (ME5053) (*recA1*...
endA1 gyrA96 thi-1 hsdR17(λK− mK−) supE44 realA1) was the host for recombinant pCA24N plasmids expressing His-tagged proteins (NBRP, National BioResource Project). Bacterial cells were routinely grown at 37°C in Luria-Bertani broth (LB). In bleomycin challenge experiments, cells were grown in minimal medium with 0.5% casein acid hydrolysate (CAA) as carbon source (Pellicer et al., 2003). Growth was monitored by measuring the optical density at 600 nm (OD600). When required, tetracycline (12.5 µg/ml), chloramphenicol (30 µg/ml) or ampicillin (100 µg/ml) was added to the medium.

2.2. Recombinant DNA techniques and site-directed mutagenesis

Bacterial genomic DNA was obtained using the Wizard Genomic DNA purification kit (Promega), and plasmid DNA was prepared using the Wizard Plus SV Midipreps DNA purification system (Promega). DNA manipulations were performed essentially as described elsewhere (Sambrook and Rusell, 2001). DNA fragments were amplified by PCR using *E. coli* chromosomal DNA as a template. PCR reactions were performed with Taq DNA polymerase or *pfu* DNA polymerase under standard conditions. DNA sequencing was carried out with an automated ABI 377 DNA sequencer and fluorescent dye termination methods.

2.3. Cloning of recombinant V5-tagged GAPDH

To construct recombinant GAPDH-V5, the pBAD TOPO® TA Expression Kit (Invitrogene) was used. This system provides a highly efficient, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector for regulated expression in *E. coli*. Expression is driven by the *araBAD* promoter, thus
induction of the cloned gene is achieved by the addition of L-arabinose to the culture medium. The *gapA* gene from strain MC4100 was amplified by PCR with primers *gapA*-pBAD-fw (TAGGGTGGAATATATGACTATCAAAGTAGG) and *gapA*-pBAD-rv (TTTGGA GATGTGAGCGATCAGG). To express GAPDH with its native N-terminal end (without the N-terminal leader), the forward primer was designed with an in-frame stop codon (TAG, underlined) at the 5’-end followed by the native *gapA* sequence including the ribosome binding site and the start codon (in bold). The *gapA* stop codon was removed from the reverse primer to allow expression of GAPDH fused at its C-terminal end to the V5 epitope. The PCR fragment was cloned into plasmid pBAD-TOPO (Invitrogen) and after transformation of strain TOP10, recombinant colonies were selected on LB-ampicillin plates. Plasmid DNA was sequenced to ensure that the fragment was inserted in the correct orientation and that no mutations were introduced. The recombinant plasmid was named pBAD-GapA.

2.4. *In vivo* cross-linking with formaldehyde

Cells of strain TOP10 bearing the recombinant plasmid pBAD-GapA were grown at 37°C in 10 ml of LB-ampicillin until the culture reached an OD$_{600}$ of 0.5. At this point L-arabinose was added at a final concentration of 0.02% to induce the expression of GAPDH-V5. After a 3 h induction, formaldehyde was added at a final concentration of 1%. Formaldehyde cross-linking was allowed to proceed for 20 min at 37°C. To stop the cross-linking reaction, glycine was added at a final concentration of 0.5 M and after 5 min at room temperature, bacterial cells were harvested by centrifugation, washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3), and resuspended in 0.4 ml of PBS containing a cocktail of protease inhibitors (Roche
Diagnostic). Cell extracts were obtained by sonic disruption of bacterial cells followed by centrifugation to remove cell debris. Protein concentration was determined using the method described by Lowry et al. (1951) with bovine serum as a standard.

2.5. Immunoprecipitation of recombinant GAPDH-V5

Purification of GAPDH-V5 cross-linked complexes was performed by immunoprecipitation using anti-V5 agarose beads (Sigma-Aldrich, Germany). To reduce unspecific interactions, the binding and washing steps were carried out under astringent conditions with RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 0.14 M NaCl, 0.01 M Tris-HCl, pH 8.0). Cell extracts were precleared with protein G-agarose beads for 10 min at 4°C. Precleared cell extracts containing 1.4 mg of protein were adjusted to a final volume of 0.8 ml with RIPA buffer and incubated with 250 µl of anti-V5 beads for 90 min at 4°C in an orbital shaker. After five washes with RIPA buffer and two additional washes with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6), the bound complexes were eluted with 0.1 M glycine (pH 2.5) for 15 min at 37°C and neutralized with a saturated Tris base solution. Protein from the eluted fractions was precipitated with acetone (at room temperature to minimize SDS precipitation), rinsed three times in acetone, and resuspended in loading buffer. Samples were then heated for 30 min at 95°C to reverse the formaldehyde cross-links. Proteins were separated on a 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and visualized by Sypro® Ruby staining.

Immunoprecipitation experiments with anti-V5 agarose beads were also carried out to identify proteins that interact with GAPDH without previous cross-linking. In this case, precleared cell extracts containing 1.4 mg of protein were adjusted to a final volume of 0.8
ml with PBS buffer and incubated with anti-V5 beads as described above. After five
washes with PBS buffer, proteins were eluted with 0.1 M glycine (pH 2.5) for 15 min at
37°C, neutralized and precipitated by incubation on ice with 10% trichloroacetic acid
(TCA). The protein pellet was washed in 90% (v/v) ice-cold acetone, air-dried and
suspended in rehydration buffer (9M urea, 4% CHAPS, 50 mM dithiothreitol, 0.5%
immobilized pH gradient buffer and traces of bromophenol blue) and processed for two-
dimensional gel electrophoresis.

2.6. Two-dimensional gel electrophoresis

2D gel electrophoresis was performed using the Protean IEF-Cell (Bio-Rad). Appropriate volumes of protein samples (5 µg of purified recombinant GAPDH) were
diluted in 125 µl of rehydration buffer (9 M urea, 4% CHAPS, 50 mM dithiothreitol, 0.5%
immobilized pH gradient buffer and traces of bromophenol blue). Isoelectric focusing was
performed in immobilized pH 3-10 or pH 5-8 gradient strips (BioRad). Second dimension
SDS-PAGE was performed on 12.5% acrylamide gels that were processed for
immunoblotting analysis. Parallel gels were silver-stained to visualize total protein.

2.7. Mass spectrometry of proteins

Following SDS-PAGE, protein bands of interest were excised from the gel and
sequentially washed with 25 mM ammonium bicarbonate and acetonitrile. These samples
were then reduced (10 mM DTT, 30 min at 56°C), alkylated (55 mM iodoacetamide, 15
min at 21°C) and digested overnight with trypsin (Tripsin Gold, Promega) at 37°C in an
automatic Investigator ProGest robot (Genomic Solutions). Peptides were extracted with
10% formic acid and analysed by combined liquid chromatography / tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF) (CapLC, Micromass-Waters) at the Barcelona Science Park (PCB) Proteomics Platform. Data were generated in PKL file format and submitted to a MASCOT server for database searching.

Protein spots from 2D gels were analysed using the liquid chromatograph nanoAcquity (Waters) coupled to a mass spectrometer (OrbitrapVelos, Thermo Scientific). Raw data were obtained with Thermo Xcalibur (v.2.1.0.1140) and used for database searching with the Proteome Discoverer software package (v.1.2.0.124).

2.8. Expression and purification of recombinant proteins

Recombinant GAPDH and Gph were expressed and purified using the Glutathione-S-transferase (GST) gene fusion system with recognition sites for factor Xa cleavage as described in detail previously (Egea et al., 2007; Pellicer et al., 2003). When indicated, Gph was expressed as His6-Gph from the ASKA clone JW3348 (NBRP, E. coli Strain National BioResource Project) and purified under native conditions with Ni2+-nitrilotriacetic acid (NTA) resin (Qiagen), as described elsewhere (Kitagawa et al., 2005). The same procedure was applied for expression and purification of the ATP synthase β subunit (His6-AtpD) and the ATP synthase α subunit (His6-AtpA) from the ASKA clones JW3710 and JW3712, respectively. Due to the low level expression of soluble His6-AtpD, purification of this protein was achieved from a 100 ml culture of strain AG1 after overnight induction with 0.1 mM IPTG at 20ºC. Cell pellets were suspended in 1 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and sonicated on ice. The cell lysate was centrifuged at 15000 g and the
supernatant incubated with 0.1 ml of Ni$^{2+}$-NTA resin for 1 h at 4°C with gentle shaking. After loading the mixture into a column, the resin was washed with the same buffer but containing 20 mM imidazole. Recombinant proteins were eluted with 0.1 ml of elution buffer containing 300 mM imidazole, followed by SDS-PAGE and immunoblotting analysis.

2.9. Immunoblotting analysis

For Western blot analysis, protein samples were separated on 10% SDS-PAGE and transferred to a HyBond-P polyvinylidene difluoride (PVDF) membrane by using a Bio-Rad MiniTransblot apparatus. The membrane was blocked in PBS-0.05% Tween-20 and 5% skimmed milk (blocking solution) for 1 h at room temperature, incubated with primary specific antibodies against GAPDH (Egea et al., 2007) (1:5,000 dilution in blocking solution) for 16 h at 4°C, washed four times with PBS-0.05% Tween-20 and incubated with the secondary antibody (donkey anti-rabbit immunoglobulin horseradish peroxidase-linked, diluted 1:15,000 in blocking solution). Incubation with the secondary antibody was omitted when anti-V5 (Invitrogen; 1:15,000 dilution) or anti-GST (GenScript; 1:5,000 dilution) horseradish peroxidase-linked antibodies were used as primary antibody. The protein-antibody complex was visualized using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

Binding of GAPDH to Gph was analysed by Far-Western assays (overlay immunoblotting). In this case, purified GAPDH was subjected to 2D gel electrophoresis as described above. After being blocked overnight with 1% gelatin in TBS buffer, the membrane was incubated with purified Gph (5 µg/ml) diluted in the same blocking buffer.
for 3 hours and then washed four times in TBS-0.05% Tween-20. To visualize interaction of GAPDH with Gph, the membrane was incubated with anti-Gph specific antibodies (1:5,000 dilution TBS-1% gelatin-0.05% Tween-20) for 16 h at 4°C, and processed as described above using the ECL Western blotting kit to visualize the reactive spots. The incubation step with purified Gph was omitted as negative control.

The anti-Gph antibodies used in this study were purified by affinity chromatography. For this purpose, purified Gph was covalently linked to Ultralink™ Immobilized DADPA on 3 M Emphase Biosupport medium AB1 gel (Pierce) using water-soluble 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-HCl. To purify the anti-Gph immunoglobulin, 3 ml of the globulin fraction partially purified from the Gph-antisera (Pellicer et al., 2003) was applied to the DADPA-Gph affinity column and the bound antibodies were eluted with 0.1 M glycine buffer (pH 2.5). Collected fractions were neutralized with Tris solution (pH 10) and dialysed overnight against PBS.

2.10. Bleomycin cell treatment

Cells of strain JA210 bearing plasmid pGEX-Gph (Pellicer et al., 2003) were grown aerobically in minimal medium with 0.5% CAA to mid-exponential phase (OD600 of 0.5). After a 2 hour induction of GST-Gph expression with 0.5 mM IPTG, the culture was treated with 8 µg/ml bleomycin in the presence of 50 µM FeSO4. At different times, aliquots of the culture were collected, washed twice with minimal medium and processed to obtain the cell extracts. Interaction between Gph and GAPDH was evaluated by pull-down experiments followed by Western blot analysis with antibodies against GAPDH. Survival was estimated by diluting cells in the same medium, followed by plating on LB-ampicillin
plates to determine the number of colony forming units (CFUs). Bleomycin sulphate (Almirall Prodesfarma, Barcelona, Spain) was freshly prepared with 50 mM phosphate buffer (pH 7.5). FeSO₄ solutions were prepared immediately before use.

3. Results and discussion

3.1. Immunoaffinity purification of proteins cross-linked to GAPDH-V5 and their identification by mass spectrometry

Cultures of TOP10 cells expressing V5-tagged GAPDH were treated with formaldehyde and the GAPDH-V5 containing complexes were analysed in the cell extracts by Western blotting with either anti-V5 or anti-GAPDH antibodies. As control, TOP10 cells without the recombinant pBAD-GapA plasmid were processed in parallel. As shown in Fig. 1A, protein bands ranging in size from 70 kDa to 160 kDa were only detected above recombinant GAPDH-V5 (37 kDa) in cells treated with the cross-linker. The most apparent bands corresponded to high molecular protein complexes in accordance with the native GAPDH tetramer structure. The presence of protein complexes with a molecular mass lower than 160 kDa indicates that other oligomeric or monomeric GAPDH-V5 forms were being cross-linked with *E. coli* proteins. To set up the conditions for cross-linking reversal, cell extracts were incubated at 65°C or 95°C in loading buffer and at the indicated times, reversion of the cross-links was analysed by Western blotting with anti-V5 antibodies (Fig. 1B). The high molecular mass complexes produced by formaldehyde treatment were no longer detected after 30 min incubation at 95°C. These conditions were selected for further experiments.
In order to identify the proteins that were cross-linked to GAPDH-V5, precleared cell extracts obtained from TOP10 (pBAD-GapA) cells challenged with formaldehyde were incubated with anti-V5 beads. After astringent washing steps, bound material was eluted as described in section 2.5. Cell extracts obtained from non formaldehyde-treated TOP10 (pBAD-GapA) cells were processed in parallel. Purified complexes were concentrated by acetone precipitation, resuspended in loading buffer and processed to reverse formaldehyde cross-links. Proteins were separated by SDS-PAGE. Staining with Sypro® Ruby revealed several proteins in the cross-linked sample that were not visible in the lane corresponding to non-treated cells (Fig. 2). These differential proteins were selected for further analysis. In the absence of formaldehyde treatment, co-purification of native GAPDH (band 2) with recombinant GAPDH-V5 (band 3) under astringent conditions (Fig. 2, - FA lane, Table 1) indicated a strong interaction of GAPDH monomers compatible with the stable tetrameric structure reported for this protein.

Twelve major bands were excised from the cross-linked sample (Fig. 2, + FA lane) and digested with trypsin. Peptides were extracted and analysed by combined liquid chromatography / tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF). Database searching in MASCOT resulted in the identification of 16 proteins, each of which matched to at least 3 unique peptide sequences (Table 1). Among the identified proteins, there were several metabolic enzymes, chaperones and factors involved in protein synthesis. Four proteins (trigger factor, DnaK, Gph and aldehyde dehydrogenase (AldA)) also appeared as candidates for interaction with GAPDH in other studies of *E. coli* or in databases such as DIP (database of interacting proteins) or IntAct. The chaperones DnaK (P0A6Y8) and trigger factor (P0A850) were identified in large-scale analyses (Arifuzzaman et al., 2006; Butland et al., 2005). Trigger factor interacts with the large
ribosomal subunit near the peptide exit channel and binds to nascent polypeptides to assist
cotranslational protein folding. Therefore, interaction of GAPDH-V5 with this chaperone is
likely to occur during the synthesis of the recombinant polypeptide. In addition, trigger
factor has been shown to prevent aggregation and promote refolding of denatured GAPDH
\textit{in vitro} (Huang et al., 2000). GAPDH has also been identified as a substrate of DnaK
(Deuerling et al., 2003). Both trigger factor and DnaK share a common substrate pool \textit{in
vivo} and cooperate to ensure proper folding of the cytosolic proteins (Deuerling et al., 1999,
2003). Thus, interaction between the synthesized recombinant protein and these chaperones
is expected to occur in cells grown at 37ºC.

GAPDH was identified as a putative interacting partner of Gph (P32662) in the
large-scale study performed by Arifuzzaman et al. (2006). In this case, the interaction was
observed with His\textsubscript{6}-Gph used as the bait in pull-down experiments. Regarding the
interaction with AldA (P25553), information available on the IntAct database
\url{http://www.ebi.ac.uk/intact/pages/interactions/} interactions) corroborates our results. Both
GAPDH and AldA were identified together with L-asparaginase-2 (P00805) as partners of
a protein complex resolved by native-PAGE.

The other proteins identified in this study as putative partners of GAPDH
interactions (Table 1) have not been experimentally isolated in previous studies. However,
some of them are predicted to be functional partners of GAPDH in the STRING interaction
network (\url{http://string-db.org/newstring_cgi/show_network_section.pl}). Enolase and
transketolase belong to this group. Other proteins may establish weak or transient
interactions with GAPDH, being captured in our experimental conditions by formaldehyde
cross-linking. Alternatively, some interactions may be indirect through association of these
proteins as partners of multiprotein complexes.
3.2. Identification of proteins that co-purify with GAPDH-V5 in the absence of cross-linking

Immunoprecipitation experiments with anti-V5 agarose beads were also carried out to identify proteins that interact with GAPDH without previous cross-linking. Precleared cell extracts were processed as described in section 2.5. Purified proteins were concentrated by TCA precipitation, resuspended in rehydration buffer and processed for 2D gel electrophoresis. In addition to the dominant spots corresponding to recombinant GAPDH-V5 and native GAPDH forms, staining with Sypro® Ruby also revealed other proteins that co-purified with GAPDH-V5 under these conditions (Fig. 2B). An immunoprecipitation experiment starting with precleared cell extracts of TOP10 cells was performed as control. In the absence of a V5-tagged protein, no apparent spots were visible (not shown), thus ruling out co-purification of these proteins through interactions with the affinity matrix.

Ten spots were excised from the 2D gel (Fig. 2B) and analysed by LC-MS/MS, in this case using a high sensitivity OrbitrapVelos mass spectrometer that enables identification of proteins even at very low amounts. Results are summarized in Table 2. Among these proteins, the ATP synthase β subunit (AtpD) and the porin OmpC, both displaying high scores in this analysis, were also identified as proteins interacting with GAPDH after formaldehyde cross-linking (Table 1). Regarding the ATP synthase α subunit (AtpA) and LpdA (both in spot 1), although these proteins were not listed in Table 1, the MS analysis performed in the cross-linking experiment revealed in both cases two peptides matching these sequences. Another protein found here to co-purify with GAPDH was GatY (30.8 kDa), the catalytic subunit of D-tagatose-1,6-bisphosphate aldolase (Brinkkötter et al., 2002). Large-scale proteomic studies performed by Burtland et al. (2005) identified the
regulatory subunit GatZ as an interacting partner of GAPDH. Neither GatY nor GatZ were
identified as interacting with GAPDH in the cross-linking experiment. However, it should
be noted that in this experiment, only proteins specifically present in the FA-treated sample
were excised and analysed by MS. In this sense, GatY should correspond to one of the non-
excised protein bands with a molecular mass around 30 kDa, also visible in the control
sample (Fig. 1A lane –FA versus +FA).

The protein displaying the highest score in this analysis was AtpD, with 40 peptides
identified that covered 85.87% of the sequence (Table 2). This ATP synthase subunit
interacts with the α subunit (AtpA) to form the catalytic domain for ATP synthesis. AtpA
also co-purified with GAPDH-V5, although the MS analysis yielded a lower score. In this
case, 29 peptides were identified that covered 61.79% of the sequence (Table 2).

To validate interaction of GAPDH with both ATP synthase subunits, pull-down
experiments with His6-tagged AtpD and AtpA proteins were performed. Recombinant His6-
AtpD and His6-AtpA were expressed and purified from the ASKA clones JW3710 and
JW3712, respectively, as described in section 2.7. Immunoblotting of the column fractions
with anti-GAPDH antibodies indicated that a fraction of GAPDH was bound and co-eluted
with either His6-AtpD or His6-AtpA (Fig. 3). In a control experiment, the cell extract
obtained from the host strain AG1 was directly applied to the Ni²⁺-NTA column and
processed in parallel as a control to confirm that the Ni²⁺-NTA resin did not bind GAPDH
to any extent (Fig. 3, right panel). It is noteworthy that even at lower bait protein
concentration the GAPDH fraction recovered in these experiments was higher in the case of
His6-AtpD. This suggests that co-purification and binding of GAPDH to AtpA may be
indirect through its interaction with the AtpD subunit. Alternatively, association of the AtpD and AtpA may be required to provide the interaction domain with GAPDH. It should be noted that in addition to AtpA, spot 1 contained other ATP-binding proteins such as AraG and RbsA. Abundance of these proteins in the spot should be very low given the low score and number of sequenced peptides (Table 2). Their identification may be attributed to the high sensitivity achieved with the analysis through use of the OrbitrapVelos mass spectrometer. All the proteins identified in spot 1 displayed similar molecular mass and pI, which is compatible with their inclusion in a single spot (Table 2). The fact that out of these five proteins, three contained ATP-binding domains suggests that GAPDH may display affinity for such domains. These kinds of interaction may point to a regulatory role of GAPDH in energy-dependent processes. One such process is cell motility. In this context, GAPDH has been suggested to play an important role in the motility of the halotolerant alga *Dunaliella salina*. In this alga, GAPDH was found in the flagellar proteome; in addition, silencing of GAPDH expression by RNAi led to reduced motility (Jia et al., 2009).

A relationship between GAPDH and cellular processes involved in energy production and conversion has been observed in *Streptococcus pyogenes* (Jin et al., 2011). Besides its cytoplasmic location, surface export of GAPDH is essential for *Streptococcus* virulence. Mutants unable to export this protein, and thus expressing higher intracellular GAPDH levels, displayed an 8-fold increase in the intracellular ATP concentration with respect to the wild-type strain. Moreover, this study revealed that intracellular levels of GAPDH in some way control the expression of certain genes (Jin et al., 2011). For instance, microarray analysis of this mutant revealed up-regulation of genes encoding proton-translocating ATPases. In this context, the interaction of GAPDH with ATP synthase subunits identified
here suggests that there are additional mechanisms in the regulatory role of GAPDH in
ATP-dependent processes.

3.3. Analysis of GAPDH interaction with Gph

Among the proteins identified as potential GAPDH interacting partners, we found the interaction with Gph to be of special interest due to its physiological role in processes linked to DNA repair. Our group showed for the first time in bacteria that this housekeeping enzyme is involved in the dissimilation of the intracellular 2-phosphoglycolate formed in the DNA repair of 3'-phosphoglycolate ends (Pellicer et al., 2003). These kinds of DNA strand break are caused by bleomycin (Povirk, 1996). In mammals, involvement of GAPDH in DNA repair processes has been well-documented (Sirover, 2005; Azam et al., 2008); however no reports on a similar function were available for prokaryotic GAPDH.

First, we aimed to determine whether GAPDH copurified with recombinant Gph. To this end, cell extracts of strain BL21 bearing plasmid pGEX-Gph, and therefore expressing GST-Gph (Pellicer et al., 2003), were applied to a glutathione-sepharose 4B resin. Cell extracts expressing the tag protein GST were processed in parallel as control. After extensive washing, elution of GST or GST-Gph was performed with glutathione elution buffer. Western blot analysis of the eluted fractions indicated that GAPDH co-purified with GST-Gph but not with GST (Fig. 4A). In a parallel experiment, elution of Gph from the affinity resin was achieved by incubation with factor Xa. Western blot analysis confirmed co-elution of GAPDH (Fig. 4B). In the eluted fractions, in addition to Gph (27 kDa), two other proteins were visible. The 34 kDa protein is one of the factor Xa subunits. The 35 kDa protein was excised from the gel and identified as GAPDH by MS. Moreover, pull-
down experiments using His<sub>6</sub>-Gph expressed in strain AG1 from the ASKA clone JW3348 as bait confirmed co-purification of GAPDH with Gph (not shown).

Overall these results confirmed interaction of GAPDH with Gph. However, when purified GAPDH was applied to an immobilized GST-Gph column, GAPDH was scarcely retained (not shown). Most of the GAPDH protein appeared in the flow-through fraction, suggesting that interaction between these proteins may either depend on other cellular factors (proteins or ligands) or on post-translational modifications of these proteins.

The presence of multiple covalently modified forms of GAPDH (Aguilera et al., 2009; Egea et al., 2007) prompted us to analyse whether the Gph binding activity is specifically linked to one of these forms. This was determined by Far-Western experiments in which purified GAPDH was subjected to 2D gel electrophoresis. Once electroblotted, the membrane was reacted with purified Gph followed by incubation with specific anti-Gph antibodies. Of the multiple GAPDH spots, only one was visualized (Fig. 3C), indicating that Gph mainly interacts with a specific form of GAPDH. These results provide evidence of a direct interaction between both proteins, and indicate that it depends on a given post-translational modification of GAPDH.

Since human GAPDH has been shown to be involved in repairing the DNA damage generated by bleomycin or alkylating agents (Azam et al., 2008), we examined whether Gph-GAPDH interacting complexes increased in cells challenged with bleomycin. To this end, induced exponential cultures of the gph mutant strain JA210 harbouring plasmid pGEX-Gph were treated with 8 µg/ml bleomycin in the presence of 50 µM FeSO<sub>4</sub>, conditions that increase the number of DNA strand breaks to be repaired. At different times, aliquots of the culture were collected and processed to obtain the cell extracts. After
30 min exposure to bleomycin, the percentage of viable cells was found to be around 30%.

Cell extracts were incubated with glutathione-sepharose 4B resin in PBS buffer. After extensive washing, GST-Gph was eluted with SDS-PAGE loading buffer. To evaluate GAPDH association, fractions were analysed by Western blotting with antibodies against GAPDH. For normalization, the same fractions were analysed in parallel with anti-GST antibodies (Fig. 5). In these experiments, the total amount of protein applied to the electrophoresis gel was lower than that used in the pull-down experiments presented in Fig. 3 in order to avoid saturation of the luminescent signal in the immunoblotting analysis. As shown in Fig. 5, the number of GAPDH molecules in complex with Gph increased in bleomycin-treated cells in a time-dependent manner. Overall, these results suggest that interaction between both GAPDH and Gph is triggered under activated DNA repair conditions. These proteins are probably partners of protein complexes involved in such processes.

Proteomic studies aimed at identifying protein interactions have great potential for suggesting new functions for a given protein. In fact, the presence of a protein in a multiprotein complex may be indicative that this protein has a second function (Jeffery, 2009). The study presented here may provide valuable clues to identify moonlighting functions for *E.coli* GAPDH. In this sense, the interaction with Gph points to a GAPDH role in cellular processes linked to DNA repair. In addition, interaction between these proteins may also suggest alternative GAPDH functions in quorum sensing signalling, since in enteric bacteria 2-phosphoglycolate is also generated as a degradation product of 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of the autoinducer-2 (Taga, 2007; Xavier et al., 2007). In conclusion, this study may contribute to the identification of further moonlighting functions of *E. coli* GAPDH at the intracellular level.
Acknowledgments

This research was supported by grant BFU 2010-22260-C02-01 from the Spanish Ministry of Education and Science to L.B. We acknowledge NBRP-\textit{E.coli} at the National Institute of Genetics for providing strain AG1 and the ASKA clones JW3348, JW3710 and JW3712. We thank E. Oliveira and M.A Odena for technical support in proteomic analysis.
References


Butland, G., Peregrin-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine,


Colussi, C., Albertini, M.C., Coppola, S., Rovidati, S., Galli, F., Ghibelli, L., 2000. H$_2$O$_2$-Induced block of glycolisis as an active ADP-ribosylation reaction protecting cells from apoptosis. FASEB J. 14, 2266-2276.


Legends to figures

Fig. 1. *In vivo* crosslinking of GAPDH-V5. TOP10 cells expressing GAPDH-V5 were incubated with 1% formaldehyde for 20 min (FA +) or left untreated (FA -). TOP10 cells without the recombinant plasmid pBAD-GapA were processed in parallel as control. (A) Cell extracts were analyzed by Western blotting with anti-V5 or anti-GAPDH specific antibodies. (B) Cell extracts obtained from TOP10 cells harbouring plasmid pBAD-GapA after formaldehyde cross-linking were incubated at the indicated conditions to reverse cross-links. Reversion was analyzed by Western blotting with anti-V5 antibodies.

Fig. 2. Immunoaffinity purification of GAPDH-V5 interacting proteins. (A) Purification of GAPDH-V5 interacting proteins after formaldehyde cross-linking. TOP10 cells expressing GAPDH-V5 were incubated with 1% formaldehyde for 20 min (+ FA) or left untreated (- FA). GAPDH-V5 complexes were purified from the corresponding cell extracts under astringent conditions using anti-V5 agarose beads. The purified complexes were incubated at 95ºC for 30 min to reverse cross-links and then separated by SDS-PAGE. Proteins were visualized by Sypro® Ruby staining. Twelve protein bands visible in the + FA lane (labelled by white numbers) were excised from the gel and analyzed by MS. M, molecular mass marker. (B) Two-dimensional analysis of proteins immunoprecipitated with GAPDH-V5 in the absence of formaldehyde cross-linking. Proteins were visualized by Sypro® Ruby staining. Ten spots (labelled by white numbers) were excised from the gel and analyzed by MS.
Fig. 3. Pull-down experiments showing binding of GAPDH to the ATP synthase α (AtpA) and β (AtpD) subunits. Cell extracts obtained from induced cells of strain AG1 bearing ASKA clone JW3710 (expressing His$_6$-AtpD) or ASKA clone JW3712 (expressing His$_6$-AtpA) were applied to a Ni$^{2+}$-NTA column. After extensive washing, elution of recombinant proteins was achieved with 300 mM imidazole (elution buffer). As a control, AG1 cell extract was applied to the Ni$^{2+}$-NTA column and processed in parallel (right panel). Column fractions (FT: flow-through; W: wash; E: elution) were analyzed either by Coomassie blue staining (upper panel) or by Western blot with anti-GAPDH antibodies.

Fig. 4. Binding of GAPDH to Gph. (A) Pull-down experiments showing binding of GAPDH to Gph. Cell extracts (0.5 ml; 10 mg/ml) obtained from induced cells of strain BLB21(DE3) bearing plasmid pGEX (expressing GST) or plasmid pGEX-Gph (expressing GST-Gph) were passed over a glutathione-sepharose 4B column. After extensive washing, elution of GST or GST-Gph was achieved with glutathione elution buffer. Column fractions (FT: flow-through; W: wash; E: elution) were analyzed either by Coomassie blue staining (upper panel) or by Western blot with anti-GAPDH antibodies. (B) Co-purification of GAPDH with Gph. Gph was purified by affinity chromatography from induced cell extracts of strain BLB21(DE3) bearing plasmid pGEX-Gph (expressing GST-Gph). In this case, elution of Gph was achieved by factor Xa digestion. The eluted fractions were analyzed either by Coomassie blue staining (upper panel) or by Western blot with anti-GAPDH antibodies. (C) Far-Western analysis of binding of soluble Gph to PVDF-immobilized GAPDH. Samples containing 5.0 µg of purified GAPDH were
subjected to 2D gel electrophoresis, and the gel was either silver-stained (first panel) or
electroblotted. The PVDF membrane was reacted with purified Gph (5 µg/ml),
subsequently incubated with antibodies against anti-Gph and processed to visualize the
reactive bands (second panel). The third panel shows the result of this analysis when the
incubation step with Gph was omitted.

Fig. 5. Pull-down experiments to analyze interaction of GAPDH with Gph in cells
challenged with bleomycin. Induced cells of strain JA210 bearing plasmid pGEX-Gph
(expressing GST-Gph) were challenged with 8 µg/ml bleomycin-50 µM de FeSO₄. At the
indicated times, cell extracts were obtained. Samples were incubated with glutathione-
sepharose 4B resin. After extensive washing, elution of GST-Gph was achieved with
loading buffer 1x. Fractions were analyzed by Coomassie blue staining and by Western blot
with anti-GAPDH or anti-GST antibodies.
<table>
<thead>
<tr>
<th>Band</th>
<th>Protein</th>
<th>Accession</th>
<th>kDa</th>
<th>MASCOT score</th>
<th>Peptides sequenced</th>
<th>Function</th>
<th>Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-ribose binding protein (RbsB)</td>
<td>P02925</td>
<td>28.5</td>
<td>236</td>
<td>5</td>
<td>D-ribose transport</td>
<td>Periplasm</td>
</tr>
<tr>
<td>1</td>
<td>Phosphoglycolate phosphatase</td>
<td>P32662</td>
<td>27.4</td>
<td>99</td>
<td>3</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH</td>
<td>P0A9B2</td>
<td>36.1</td>
<td>447</td>
<td>11</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>2</td>
<td>OmpC</td>
<td>P06996</td>
<td>40.4</td>
<td>91</td>
<td>3</td>
<td>Porin</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>3</td>
<td>GAPDH-V5</td>
<td>--</td>
<td>--</td>
<td>347</td>
<td>12</td>
<td>Recombinant protein</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>EF-Tu</td>
<td>P0CE47</td>
<td>43.3</td>
<td>503</td>
<td>14</td>
<td>Protein synthesis</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>5</td>
<td>Enolase</td>
<td>P0A6P9</td>
<td>45.6</td>
<td>238</td>
<td>5</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>6</td>
<td>Tryptophanase</td>
<td>P0A853</td>
<td>53.1</td>
<td>183</td>
<td>5</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>6</td>
<td>ATP synthase β subunit</td>
<td>P0ABB4</td>
<td>50.3</td>
<td>98</td>
<td>3</td>
<td>ATP synthesis</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>7</td>
<td>Aldehyde dehydrogenase (AldA)</td>
<td>P25553</td>
<td>52.4</td>
<td>284</td>
<td>6</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>7</td>
<td>Trigger factor</td>
<td>P0A850</td>
<td>49.0</td>
<td>115</td>
<td>4</td>
<td>Chaperone</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>7</td>
<td>Pyruvate kinase</td>
<td>P0AD61</td>
<td>51.6</td>
<td>97</td>
<td>3</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>8</td>
<td>Non identified</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>9</td>
<td>DnaK</td>
<td>P0A6Y8</td>
<td>69.1</td>
<td>510</td>
<td>10</td>
<td>Chaperone</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>10</td>
<td>Transketolase</td>
<td>P27302</td>
<td>72.4</td>
<td>105</td>
<td>5</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>11</td>
<td>Aconitase</td>
<td>P25516</td>
<td>94.0</td>
<td>135</td>
<td>3</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>12</td>
<td>Non identified</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
### Table 2.
Proteins that copurified with GAPDH-V5 in the absence of formaldehyde cross-linking

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Accession</th>
<th>kDa</th>
<th>pl</th>
<th>Score</th>
<th>Peptides sequenced</th>
<th>Function</th>
<th>Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATP synthase α subunit</td>
<td>P0ABB0</td>
<td>55.2</td>
<td>6.13</td>
<td>608.59</td>
<td>29</td>
<td>ATP synthesis</td>
<td>Membrane bound</td>
</tr>
<tr>
<td>1</td>
<td>Anthranilate synthase, component II</td>
<td>P00904</td>
<td>56.8</td>
<td>6.52</td>
<td>74.04</td>
<td>10</td>
<td>Trp biosynthesis</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>1</td>
<td>ATP-binding protein AraG</td>
<td>P0AAF3</td>
<td>55.0</td>
<td>6.38</td>
<td>59.09</td>
<td>10</td>
<td>L-Arabinose transport</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>1</td>
<td>ATP-binding protein RbsA</td>
<td>P04983</td>
<td>55.0</td>
<td>6.14</td>
<td>50.91</td>
<td>11</td>
<td>Function</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>1</td>
<td>Dihydrolipoyl dehydrogenase (lpdA)</td>
<td>P0A9P0</td>
<td>50.7</td>
<td>6.15</td>
<td>49.10</td>
<td>11</td>
<td>Pyruvate DH component</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>2</td>
<td>Non-identified</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>ATP synthase β subunit</td>
<td>P0ABB4</td>
<td>50.3</td>
<td>5.01</td>
<td>1150</td>
<td>40</td>
<td>ATP synthesis</td>
<td>Membrane bound</td>
</tr>
<tr>
<td>4</td>
<td>OmpF</td>
<td>P02931</td>
<td>39.3</td>
<td>4.96</td>
<td>156.9</td>
<td>19</td>
<td>Porin</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>5</td>
<td>OmpC</td>
<td>P06996</td>
<td>40.3</td>
<td>4.82</td>
<td>445.05</td>
<td>31</td>
<td>Porin</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>5</td>
<td>GAPDH</td>
<td>P0A9B2</td>
<td>35.5</td>
<td>7.11</td>
<td>153.96</td>
<td>19</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>6</td>
<td>GAPDH</td>
<td>P0A9B2</td>
<td>35.5</td>
<td>7.11</td>
<td>629.11</td>
<td>29</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>7/9/10</td>
<td>GAPDH proteolysis fragments</td>
<td>P0A9B2</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>8</td>
<td>D-tagatose-1,6-bisphosphoaldolase, GatY</td>
<td>P0C8J6</td>
<td>30.8</td>
<td>6.34</td>
<td>259.55</td>
<td>21</td>
<td>Metabolic enzyme</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>
FIGURE 1

(A) TOP1GAPDH-V5 TOP10 GAPDH-V5 TOP10
FA - + - + - + - +
180 160 140 120 100 80 60 40 20 0
GAPDH-V5 GAPDH
Anti-V5 Anti-GAPDH

(B) GADPH-V5
Anti-V5
FIGURE 2

(A)

(B)
FIGURE 4

(A) GST GST-Gph

FT W E1 E2 FT W E1 E2

GST-Gph →
GST →

Anti-GAPDH

(B) KDa

E1 E2

GAPDH (35 kDa)
Gph (27 kDa)

Anti-GAPDH

(C) Silver staining

GAPDH

Anti-Gph

GAPDH (+ Gph)

GAPDH (- Gph)
FIGURE 5

![Image of a gel electrophoresis experiment with bands labeled for GST and GAPDH proteins at different time points.](image-url)