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Glucose 6-phosphate and alcohol dehydrogenase activities are components of dynamic macromolecular depots structures

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ABSTRACT

Background: Membrane-associated respiratory complexes, purinosome and many intracellular soluble activities have reported to be organized in dynamic multi-component macromolecular complexes using native PAGE, 2D SDS-PAGE, electron and systematic microscopy and genome-wide GFP fusion library.
Methods: In-gel staining assays, SDS-PAGE and LC–MSMS techniques were performed on cellular extracts to analyze, isolate and identify the proteins associated with glucose 6-phosphate dehydrogenase (G6PDH) and fermentative alcohol dehydrogenase (ADH) I isoform in both *Kluyveromyces lactis* and *Saccharomyces cerevisiae* yeasts.
Results: Analysis of LC–MSMS data showed that a large number of components, belonging to glycolysis, pentose phosphate, folding and stress response pathways, were associated with G6PDH and Adh1 putative complexes and that a number of these proteins were identical in either network in both yeasts. However, comparison of in-gel staining assays for hexokinase, phosphoglucoisomerase, acetaldehyde dehydrogenase, ADH and G6PDH showed that, despite their identification in these structures, functional localization of these activities varied according to growth conditions and to NAD(P)$^+$/NAD(P)H redox ratio.
Conclusions: Reported data show that intracellular proteins are organized in large dynamic 'depots' and the NAD(P)$^+$/NAD(P)H redox balance is one of the major factors regulating the assembly and the re-assortment of components inside the different metabolic structures.
General significance: The aim of this work is directed towards the comprehension of the mechanisms involved in the assembly, organization, functioning and dynamic re-assortment of cellular components according to physiological and/or pathological conditions.

1. Introduction

Most cellular processes are carried out by protein complexes [1], which provide increased reaction efficiency, metabolite channeling and coordinated control of reaction rates. Many complexes have been characterized and the 3D structure determined for a few of them: the fatty acid synthase complex is just an example [2]. Many, if not all, complexes in turn can assemble in dynamic multi-component macromolecular networks, called "depots" [3,4]. In these networks, individual components can be transiently held and released, migrate away from a complex or participate to multiple spatial and functional distinct depots according to cellular needs. Moreover, each component may be inactive in a complex and become functional upon release, or may have the same or different function in and out of the complex [3]. Many reports have provided evidence for the existence of these supra-molecular structures, among them tRNA multisynthetase complex, the purinosome and the ribosome [5–7]. Experimental studies using affinity purification and mass spectrometry aiming at to define the interactome in *Saccharomyces cerevisiae* have shown that in this organism more than 500 protein complexes exist and most of these complexes have a component in common with at least one other multi-protein assembly [8,9]. The organization of macromolecules in dynamic depots may play important roles during the cell cycle [10] and also during stationary phase, when a large number of metabolic proteins form reversible macroscopic foci inside quiescent cells [11]. Re-entry into proliferating conditions when nutrients are made available requires a large-scale reprogramming of the metabolic and replicatory machinery held in poised state during quiescence. Systematic microscopy tracking of proteins and genome-wide GFP fusion library has shown that in this condition a large-scale reorganization of components in the complexes occurs. Several tagging methods have been used to detect and analyze single multi-protein complex speculating that the large-scale reorganization of components may

Abbreviations: ADH, alcohol dehydrogenase; Eno, enolase; G6PDH, glucose 6-phosphate dehydrogenase; PPP, pentose phosphate pathway; LC–ESI-MSMS, liquid chromatography–electrospray ionization-tandem mass spectrometry

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function a) to improve catalytic efficiency, b) as storage depots and c) to aggregate dysfunctional/unfolded proteins [11,12].

In the present work we used native polyacrylamide gels electrophoresis (PAGE), staining assays, SDS-PAGE, analysis of mutants and LC–MSMS techniques to separate, isolate and identify the proteins associated with glucose 6-phosphate dehydrogenase (G6PDH) and the fermentative alcohol dehydrogenase (ADH) I isoform. These two key enzymes, located at crucial glycolysis/PPP and glycolysis/fermentation routes, provide NADPH for biomass synthesis/response to stress and to reoxidize the NADH necessary to feed glycolytic flux, respectively, in both *Kluyveromyces lactis* and *S. cerevisiae* yeasts.

G6PDH, an evolutionary conserved protein [13,14], catalyzes the rate-limiting NADPH producing step of the pentose phosphate pathway (PPP) [15]. In the yeast *K. lactis* this enzyme has been detected, on native PAGE, as two differently migrating forms [16] representing different oligomeric states of the enzyme determined by cytosolic accumulation of NAD(P)H [17].

G6PDH oligomers, as well as Adh1, were singularly cut, eluted and purified from native gels and then analyzed on SDS-PAGE. Surprisingly, a large number of almost identical proteins in different growth conditions were found to be associated with G6PDH and Adh1 in both *K. lactis* and *S. cerevisiae* yeasts. These proteins were identified by mass spectrometry as glycolytic, PPP, folding, stress response related proteins and other components of the cellular/cytoskeleton structures. The presence of different intracellular complexes containing a relevant number of common components suggested the participation of these proteins in multiple functional distinct networks. Specific staining assays for components of these macromolecular structures led us to the conclusion that they are organized into dynamic "depots", whose components can be active or inactive depending on the growth under physiological/pathological conditions.

2. Materials and methods

2.1. Strains, media and culture conditions

The *K. lactis* strains used in this work are reported in Table 1. Media preparations and cultures conditions were performed as previously described [18].

2.2. ADH, ALD, G6PDH, HXK and PGI in-gel staining assays

K. lactis cell extract preparation, clear native PAGE, electrophoresis conditions and ADH and G6PDH staining assays were carried out as previously described [16,18]. G6PDH in vitro assay has been previously described [16].

In-gel staining assay for aldehyde dehydrogenase (ALD) was performed as described for ADH but with longer incubation times, with the following staining mixtures ($NADP^+$-dependent): 20 μl $NADP^+$, 30 μl NTB, 15 μl PMS, 5 μl 3 M CH_3COOK, 50 μl acetaldehyde 10% (v/v), and H_2Odd to 5 ml; (NAD^+-dependent): 30 μl NAD^+, 30 μl NTB (Nitrotetrazolium Blue), 15 μl PMS (Phenazine Methosulfate), 5 μl $MgCl_2$ 1 M, 50 μl acetaldehyde 10% (v/v), and H_2Odd to 5 ml. Stock solutions for NAD^+, $NADP^+$, NTB and PMS were as previously described [16,18].

In-gel phosphoglucose isomerase (Pgi) and hexokinase (Hxk) staining assays were performed placing, after the removal of one of the glasses, the native run gel on a polymerized polyacrylamide gel containing the staining mixture. Staining gel for PGI: 0.8 ml acrylamide/bis 40% (w/v) solution, 2.0 ml TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), 2.4 ml H_2Odd, 40 μl $NADP^+$, 40 μl NTB, 25 μl PMS, 5 μl 1 M $MgCl_2$, 40 μl fructose 6-P (100 mg/ml), 600U G6PDH (yeast), 50 μl 10% (w/v) ammonium persulfate and 6 μl TEMED. Staining gel for HXK: 0.8 ml acrylamide/bis 40% (w/v) solution, 2.0 ml TE, 2.4 ml H_2Odd, 40 μl $NADP^+$, 40 μl NTB, 25 μl PMS, 50 μl glucose (100 mg/ml); 500 μl ($NADP^+$, ATP and magnesium sulfate in triethanolamine buffer solution pH 7.6) (from r-biopharm D-glucose kit cat. 10716251035), 50 μl 10% ammonium persulfate and 6 μl TEMED.

Table 1
Yeast strains used in this study.

K. lactis	Genotype	Reference
CBS2359	*MAT**a***	CBS collection
GG1996	*MAT**a** rag2Δ*	[17]
MW179-1D	*MATα metA1 ade-T600 leu2 trpA1 ura3 lac4*	[17]
MS7-62	*MATα lysA1 argA1 adh1Δ adh2Δ adh3Δ adh4Δ*	[17]
MS7-62/ADH1	*MATα lysA1 argA1 adh2Δ adh3Δ adh4Δ*	[17]
MW270-7B	*MAT**a** metA1 leu2 ura3*	[19]
MW270-7B/eno1Δ	*MAT**a** metA1 leu2 ura3 eno1Δ*	[19]
JA6/dgr151	*MATα ade1ade2 trp1-11 adh3 rag5*	[20]
PM6-7A	*MAT**a** ade-T600 uraA1-1 adh3*	[21]
PM6-7A/rag6	*MAT**a** ade-T600 uraA1-1 adh3 rag6Δ*	[21]
PM6-7A/tpi1Δ	*MAT**a** ade-T600 uraA1-1 adh3 tpi1Δ*	[22]
MW98-8C	*Matα lysA1 argA1 ura3 rag1 rag2*	[23]
MW98-8C/pgk1Δ	*Matα lysA1 argA1 ura3 rag1 rag2 pgk1Δ*	[23]
S. cerevisiae		
BY4741	*MAT**a** hys3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf

2.3. Determination of ADH and G6PDH enzymatic activities

ADH activity was assayed by measuring the rate of NAD^+ reduction/NADH reoxidation at 340 nm in 50 mM Tris–HCl, pH 8.0 buffer containing either ethanol (200 mM) or acetaldehyde (20 mM) as a substrate. G6PDH activity was assayed by measuring the rate of $NADP^+$ reduction at 340 nm in 0.1 M Tris–HCl buffer, pH 8.8, containing 10 mM $MgCl_2$, 0.1 mM glucose-6-phosphate and 0.1 mM $NADP^+$.

2.4. Elution and analysis of stained G6PDH and Adh1 bands from native PAGE

The stained bands on native PAGE were cut and the proteins extracted from the bands with extraction devices (Millipore), concentrated and washed with TE buffer using 10 kDa MWCO ultrafiltration tools (Vivaspin, Sartorius). About 2 μg of proteins were finally yielded from 2 to 2.5 mg of total soluble proteins fractionated on native PAGE.

The G6PDH-specific band eluted from native PAGE was analyzed on size exclusion column (Superdex 200; GE Healthcare) for FPLC experiments equilibrated with Tris–glycine buffer and calibrated with ferritin (440 kDa), conalbumin (75 kDa), bovine serum albumin (67 kDa) and RNase A (13.7 kDa).

The identification of proteins within G6PDH and Adh1 native PAGE extracted bands was performed by nanoLC–ESI-MSMS by a custom service (Proteome Factory). The mass spectral data were interpreted with Mascot software. In the supplementary tables (S1–S7) we reported the results of all protein identifications, with the score (combination of scores of all observed mass spectra that can be matched to amino acid sequences within that protein), the number of identified peptides per protein, and emPAI (Exponentially Modified Protein Abundance Index), an index that indicates the protein content on the basis on the ratio of the number of observed peptides to the number of observable peptides [24]. From these data we have pulled out the proteins with high score and high emPAI presented in summary in Tables 2, 3 and 4.

2.5. SDS-PAGE analysis and western blot

Protein samples were run on a 12% SDS-PAGE and transferred onto PVDF membranes (Immobilon; Millipore) using the mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were probed with anti-G6PDH (LifeSpan Biosciences), anti-enolase (anti-Eno; Santa cruz Biotechnology), anti-triosephosphate isomerase (anti-Tpi; Nordic Immunology Laboratories) and anti-3-phosphoglycerate kinase (Molecular Probes) polyclonal antibodies.