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Quantification of proteome dynamics in *Corynebacterium glutamicum* by ^{15}N-labeling and selected reaction monitoring

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ABSTRACT

Selected reaction monitoring allows quantitative measurements of proteins over several orders of magnitude in complex biological samples. Here we present a targeted approach for quantification of 19 enzymes from *Corynebacterium glutamicum* applying isotope dilution mass spectrometry coupled to high performance liquid chromatography (IDMS–LC–MS/MS). Investigations of protein dynamics upon growth on acetate and glucose as sole carbon source shows highly stable peptide amounts for enzymes of the central carbon metabolism during the transition phase and after substrate depletion. However significant adaptations of protein amounts are observed between both growth conditions well agreeing with known changes in metabolic fluxes. Time-resolved measurements of protein expression after metabolic switch from glycolytic to gluconeogenetic conditions reveal fast responses in protein synthesis rates for glyoxylate shunt enzymes.

1. Introduction

The Gram positive microorganism *Corynebacterium glutamicum* is a widely used industrial host for the production of amino acids [1]. Intensive manipulations using classical mutagenesis as well as next generation rational design methods have produced several strains used for industrial production of L-glutamate, L-lysine and L-threonine [2]. Since its complete genome sequence has been disclosed during this progress [3,4], *C. glutamicum* has become a model organism for the group of *Corynebacterianeae* including its pathogenic close relative *Mycobacterium tuberculosis* [5,6]. The development and application of omics-technologies for transcriptomics [7–9], metabolomics [10–12] and fluxomics [13–15] already led to a sustainable quantitative database for a better system level understanding of this organism. With regard to proteomics gel based data offering limited quantitative insights to *C. glutamicum* proteome became available first [16]. Gel free systems focusing on membrane and cytoplasmic proteome adaptations to pH and salt stress [17–19] as well as L-lysine production [20] have recently been presented. Furthermore, stable isotope dilution mass spectrometry (IDMS) using ^{15}N-metabolic labeling allows reliable peptide and protein quantitation in complex biological samples without need for complete separation of the analytes [21]. Clearly, as true for mRNA levels, absolute quantification of proteins is strongly limited by the availability of specific standards, since their generation is usually very difficult, time-consuming and expensive [22,23]. However, concerning *C. glutamicum*, currently no method is available that allows for measuring relative amounts of proteins and more specifically enzymes of the central metabolism on triple quadrupole mass spectrometers which would provide the best technological platform for absolute quantification.

One of the outstanding characteristics of *C. glutamicum* is the ability to grow on organic acids such as acetate or lactate [24]. Furthermore, *C. glutamicum* is able to co-metabolize these substrates simultaneously with glucose [25]. This is in strong contrast to other bacteria like *Escherichia coli* or *Bacillus subtilis* that prefer glucose and show a diauxic growth pattern when additional organic acids are present [26,27]. Therefore, the acetate metabolism of *C. glutamicum* and its regulation has been thoroughly studied [28]. Investigations on multiple omics levels

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have pointed out a distinct modulation of the central carbon metabolism which gives a general explanation for the growth modus on gluconeogenetic substrates. The main motives of acetate metabolization described so far are: i) activation of acetate via the Acetate kinase and Phosphotransacetylase operon *ack-pta* [29,30]. ii) Increased carbon flux through TCA and newly activated glyoxylate shunt. iii) Predominant reverse anaplerotic flux through Phosphoenolpyruvate carboxykinase (PEPCk) to provide phosphoenolpyruvate for gluconeogenetic generation of biomass precursors [31]. Despite these detailed information on transcriptional changes, enzyme activities and flux distributions, no quantitative data on protein amounts are available yet, leaving a substantial gap in the description of this important substrate metabolism in *C. glutamicum*.

In this study we investigated the dynamic adaptation of the central carbon metabolism of *C. glutamicum* upon changes of growth state and carbon source by quantitative protein measurements employing selected reaction monitoring and ^{15}N labeling technique. To our knowledge this is the first report providing quantitative time-resolved protein expression patterns for *C. glutamicum* in response to carbon source availability.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Corynebacterium glutamicum ATCC 13032 was grown in CGXII minimal medium as described in [32] w/o urea and with 0.6–2% (w v^{-1}) glucose or 0.6–1.2% (w v^{-1}) acetate as the sole carbon source. Overnight precultures (30 °C, 165 rpm) in baffled shaking flasks were used to inoculate 1 ml cultivations in 48well Flowerplates® (m2p-labs, Aachen). Cultivation was performed in the BioLector® (m2p-labs, Aachen) at 30 °C, 80% humidity and 1300 rpm. Biomass, pO_2 and pH were monitored online via backscatter (CDW [g l^{-1}]=1234*BS [–]+5678) and fluorescence measurements, respectively [33]. Acetate shift experiments were performed in a 1.5 l parallel bioreactor system (DASGIP, Jülich) at 30 °C using 1 l CGXII minimal medium w/o MOPS and urea. Dissolved oxygen was regulated at 30% saturation via stirrer speed control (400–1200 rpm at 60 l h^{-1}) and pH was maintained at pH 7.0 by adding 2.5 M HCl or 4.0 M NaOH.

Isotope dilution mass spectrometry (IDMS) applying stable heavy isotopes as internal standards (IS) was used in this study to reduce errors during sample handling and measurement. For internal standard generation cells were cultivated in baffled shaking flasks using CGXII minimal media with carbon sources as stated above but $^{15}NH_4SO_4$ as the sole nitrogen source. Glucose and acetate grown IDMS cells were mixed at a ratio of 50:1. This ratio was sufficient to supplement IS with the proteins Isocitrate lyase (ICL) and Malate synthase (MS) which are highly underrepresented in glucose grown cells, but strongly expressed under acetate conditions.

2.2. Sample preparation

For LC–MS measurements 4 mg biomass (cell dry weight) were harvested from each well of BioLector cultures or shaking flask cultures by centrifugation (1 min, 4 °C, 16,000 ×*g*), immediately frozen in liquid nitrogen and stored at -80 °C until further processing. Cells thawed on ice were washed with 500 µl lysis buffer and after centrifugation done as before resuspended in 400 µl lysis buffer. Composition of lysis buffer was 50 mM potassium phosphate buffer, pH 8.0, 2 mM EDTA, 2 mM DTT, supplemented with complete protease inhibitor cocktail (1 697 498, Roche Applied Science). Cells were disrupted in a beadmill (Retsch GmbH, Hahn, Germany) using 0.1 mm glass beads 5× for 30 s at highest settings in a cold room (4 °C). Cell debris were removed (60 min, 4 °C, 16,000 ×*g*) and protein concentration of crude extracts was estimated using a Bradford assay (B6916, Sigma Aldrich) with BSA as a standard within a linear range of 0.1–1.4 mg ml^{-1}. A maximum of 50 µl of the resulting crude extracts (up to 100 µg total protein) could readily be used for tryptic digestion. For relative quantification 50 µg of ^{14}N-crude extracts and 50 µg of ^{15}N-IDMS crude extract were digested together with 1 µg trypsin in a total volume of 100 µl for 5 h at 42 °C as recommended by the supplier (T7575, Sigma Aldrich). For peptide screening, assay development and optimization ^{14}N samples were used (100 µg total protein). Peptide solutions were diluted 1:2 with MilliQ H_2O (Millipore, Merck KGaA) prior to LC–MS measurements.

2.3. LC–MS method development and measurements

A targeted approach was used for quantitative assay development. Peptides of desired enzymes with 6 to 30 aa derived from *C. glutamicum* genome sequence [3] were fragmented *in silico* using Skyline software [34] to create screening methods for ABSciex 4000QTRAP mass spectrometer. For peptide dentification the MS was used in information dependent acquisition mode were predicted MS/MS transitions triggered fragment spectra acquisitions in the linear ion trap at >800 cps (IDA-EPI). Peptides were identified using Matrix Science Mascot database search (ion score>42, $p<0.05$) and by manual revision of acquired MS/MS spectra. Most abundant transitions with m/z of product ion>m/z of precursor ion from identified spectra were chosen for further optimization of quantitative assays. For each protein three peptides were analyzed. Uniqueness of peptides was assured by BLASTp controls against a *C. glutamicum* background proteome using Skyline software. For each peptide three transitions were measured.

With injected sample volumes of 10 µl, *on column* protein amounts of up to 5.0 µg (e.g. 2.5 µg ^{14}N-sample, 2.5 µg ^{15}N-IS) were applied. These total protein amounts are within the linear range of the LC–MS system used in this study (Agilent 1100 series & ABSciex 4000 QTRAP). Peptide mixtures were separated by reverse phase HPLC prior to ESI-MS/MS measurements (guard column: 2×0.8 mm PepMax300 C18 5 µm (163942, Dionex), RP-column at 21 °C: 150×2.1 mm Ascentis Express® Peptide ES-C18 2.7 µm (53307-U, Sigma Aldrich); equilibration: 8% B (15 min); gradient B: 8–40% (60 min), 40% (3 min), 40–60% (1 min), 60% (10 min); flow: 0.1 ml min^{-1}; A: 0.1% (v v^{-1}) formic acid, B: acetonitrile+0.1% (v v^{-1}) formic acid). MS settings were: Turbo-V Source, CUR: 10 psi (N_2), IS: +5500 V, Temperature: 300 °C, Source Gas 1 and 2: 30 psi (N_2), interface heater: off, CAD: 5 psi (N_2), entrance potential 10 V, collision cell exit potential 12 V, Q1: unit resolution with IE: 1.0, Q3: unit resolution with IE: 0.3. Dwell time was 50 ms during IDA-EPI screening runs. For scheduled SRM a target scan time of 4 s was applied. Width of the detection window was 240 s centered on peptide retention times.