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The glucose transporter of *Escherichia coli*

Purification and characterization by Ni^+ chelate affinity chromatography of the $IIBC^{Glc}$ subunit

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The $IIBC^{Glc}$ transmembrane subunit of the glucose transporter of *E coli* containing a carboxy-terminal affinity tag consisting of six adjacent histidines was purified by nickel chelate affinity chromatography. The protein was constitutively overexpressed from a high copy number plasmid. 1.5 mg of 95% pure protein was obtained from 5 g (wet weight) cells. 70% of the phosphotransferase activity present in cell membranes was recovered. Adsorption to the nickel resin allows delipidation as well as rapid detergent exchange. The procedure was used to demonstrate exchange of subunits in the $IIBC^{Glc}$ dimer and it holds promise for the investigation of other protein–protein interactions

Phosphotransferase system; Glucose carrier; Ni^+ chelate chromatography; Enzyme II; *E coli*

1. INTRODUCTION

The glucose transporter of *E. coli* acts by a mechanism which couples vectorial transport with phosphorylation of the translocated substrate. It consists of two subunits, IIA^{Glc} and $IIBC^{Glc}$. $IIBC^{Glc}$ is a transmembrane protein of M_r 50,645 [1]. It consists of two structural domains, IIB and IIC. The amino-terminal IIC domain spans the membrane eight times and contains the sugar binding site [2,3]. The IIB domain is hydrophilic and accessible from the cytosol. It mediates phosphoryltransfer from the IIA subunit to the sugar substrate. A cysteine is transiently phosphorylated in this process [4]. The solubilized $IIBC^{Glc}$ subunit is a dimer. $IIBC^{Glc}$ belongs to the group of sugar specific components known as 'membrane-linked enzymes II' of the bacterial phosphotransferase system [5–7]. In addition to the sugar specific transporters, the phosphotransferase system contains two cytoplasmic proteins, enzyme I and HPr, which sequentially transfer phosphoryl groups from phospho-enolpyruvate to the IIA units.

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Abbreviations: $IIBC^{Glc}$ (*ptsG*), transmembrane subunit of the glucose transporter (*gene*); IIA^{Glc} (*crr*) cytoplasmic subunit of the glucose transporter (*gene*); $IIAB^{Man}$ (*manX*), IIC^{Man} (*manY*) and IID^{Man} (*manZ*), subunits of the mannose transporter (*genes*) $IIABC^{GlcNAc}$ (*nagE*), transporter for *N*-acetyl glucosamine (*gene*); NTA, nitrilotriacetic acid.

$IIBC^{Glc}$ can be overexpressed and has been purified in mg amounts by isoelectric focusing in a sucrose gradient and gel filtration [8]. This method is also used to purify the phosphotransferase transporters for mannose [9] and *N*-acetyl glucosamine (Erni, unpublished obeservation). However, it is laborious, expensive and it cannot be scaled up because of the limited solubility of $IIBC^{Glc}$ at high concentration. To prepare the protein amounts required for biophysical studies and crystallization a simpler method of purification is desirable. Here we show that a modified form of the $IIBC^{Glc}$ subunit can be purified in a single step and in biologically active form by Ni-chelate affinity chromatography. The procedure lends itself for the rapid exchange of detergents and it can be used for qualitative studies of protein–protein interactions in the $IIBC^{Glc}$ dimer.

Ni chelate affinity chromatography exploits the affinity of adjacent imidazole groups for Ni^{2+} complexed via nitrilotriacetic acid (NTA) to a solid agarose matrix [10,11]. The imidazole groups are introduced as a run of histidines attached to the N- or C-terminus of the protein to be purified. In contrast to immune- and ligand-affinity chromatography this method is independent of protein tertiary structure. It only requires that the histidines are accessible from the protein surface.

2. MATERIALS AND METHODS

2.1. *Construction of a ptsG expression plasmid*

The *ptsG* gene was cloned into a mutagenesis vector in order to introduce a restriction site precisely at the 3′ end of the coding region. From there the *ptsG* gene was cloned into an expression vector encod-

ing the polyhistidine sequence. Four steps were involved: (i) the *Eco*RI-*Hin*dIII fragment of pTSG31 [2] was cloned into the mutagenesis vectors pMa5–8 and pMc5–8 [12] from which the unique *Asu*II site previously had been deleted. (ii) An *Asu*II restriction site was introduced at the 3′ end of the *ptsG* coding region by gapped duplex site-directed mutagenesis with the synthetic oligonucleotide CCGTAACTTCGAATCCGTAGG. This resulted in the mutation H477F. (iii) The *Hin*dIII-*Asu*II fragment containing the 5′ non-coding and complete coding region of *ptsG*, and the expression vector pQE-12 (Qiagen, Diagen GmbH, Düsseldorf, Germany) opened with *Eco*RI and *Bam*HI were ligated after the protruding ends were blunt-ended using Klenow fragment. (iv) *E. coli* K-12 LR2-168 (*ptsG nagE manXYZ*) was transformed and plated on McConkey indicator plates containing 0.4% glucose. Plasmids were isolated from glucose fermenting colonies. The orientation of the insert was determined by restriction analysis. Plasmid pQEGH12 containing the insert in the correct orientation was sequenced. The amino acid sequence NF_{477}GSRSHHHHHH(COOH) of the $IIBC^{Glc}$ carboxy-terminus was deduced from the DNA sequence at the fusion joint.

2.2. *Expression and purification of $IIBC^{Glc}$-6H*

E. coli ZCS112L [13] transformed with pQEGH12, were grown overnight to saturation in LB medium. Membranes were prepared by rupturing of bacteria in a French pressure cell [9]. The membrane pellet was resuspended in buffer A (1 ml/g wet weight of cells, 10 mM Tris-glycine, pH 8.9, 10 mM β-mercaptoethanol, 1 mM methyl α-D-glucopyranoside). Triton X-100 was added to a final concentration of 2% with stirring at 4°C. The clarified solution was freed of insoluble material by ultracentrifugation (226,000 × *g*, 2 h 4°C). The pH of the membrane extract was adjusted to pH 8.0 with 0.5 M Na_2HPO_4 and the extract loaded onto Ni-NTA-agarose (Quiagen, Diagen GmbH, Düsseldorf, Germany) 3 ml bed volume for the membrane extract from 1 g wet weight of cells) equilibrated with buffer B (50 mM NaP_i pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 1 mM methyl α-D-glucopyranoside, 0.1% Triton X-100). Unspecifically bound proteins were eluted with buffer B of pH 8.0 and pH 6.0 (5 bed volumes each) $IIBC^{Glc}$-6H was eluted with buffer B, pH 5.0. The fractions were immediately neutralized with 1 M NaOH. The pooled fractions were concentrated in a Centricon (Amicon) to 0.5 mg/ml. 0.5 ml were purified by gel filtration (Superose 12HR 10/30 Pharmacia, 0.25 ml/min, 25 mM NaP_i pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM glucose, 0.1% Triton X-100). For delipidation experiments where phosphate determinations were required, 20 mM Tris-HCl, pH 8.0, and 20 mM sodium acetate, pH 6 and pH 5, replaced NaP_i buffers.

2.3. *Analytical methods*

Sugar phosphorylation activity was assayed by the ion-exchange method [8]. The reaction mixture contained per 0.1 ml: 100 μg egg yolk phosphatidylglycerol (Sigma), 50 mM KP_i, pH 7.5, 2.5 mM DTT, 2.5 mM NaF, 5 mM $MgCl_2$, 1 mM phospho-enolpyruvate, 0.5 mM [U-^{14}C]α methyl-D-glucopyranoside (New England Nuclear, 250 mCi/mmol, diluted to 900 cpm/nmol), 2.5 μg enzyme I, 2 μg HPr, 2 μg IIA^{Glc}. Enzyme I, HPr and IIA^{Glc} were purified from an overexpressing *E. coli* strain (Erni, unpublished). Incubation was at 37°C for 30 min. Standard methods of gel electrophoresis, electroblotting, and determination of total phosphate and protein were used [8].

3. RESULTS AND DISCUSSION

3.1. *Purification by Ni-chelate affinity chromatography*

Extension of the carboxy-terminus by ten residues six of which are histidines does not affect protein activity and stability. On the contrary, the total amount of $IIBC^{Glc}$-6H expressed from pQEGH12 is slightly higher than the amount obtained with plasmid pTSG4 [1]. The protein is expressed constitutively under the control of the *ptsG* promotor in the 270 nucleotides region upstream of the coding sequence. The vector (pQE12) born tacP promoter does not affect transcription. The carboxy-terminal extension causes a decrease of the electrophoretic mobility (Fig. 2, see below). 90% of the glucose phosphotransferase activity of crude membranes could be solubilized at pH 8.9 with 2% Triton X-100. Only 60% activity were solubilized when NTA-column buffer (pH 8.0, 300 mM NaCl, 2% Triton X-100) was used for membrane solubilization (results not shown). To match the conditions recommended for protein binding to the Ni affinity resin, the pH was lowered to pH 8.0 after the protein had been solubilized at pH 8.9. After elution of the contaminating proteins with pH 6, virtually pure $IIBC^{Glc}$-6H could be eluted with buffer of pH 5.0 (Fig. 1). A minor contaminant of 67 kDa could be removed by gel filtration chromatography (not shown). Approximately 1.3 mg of purified protein are obtained from 5 g (wet weight) of cells (Table I).

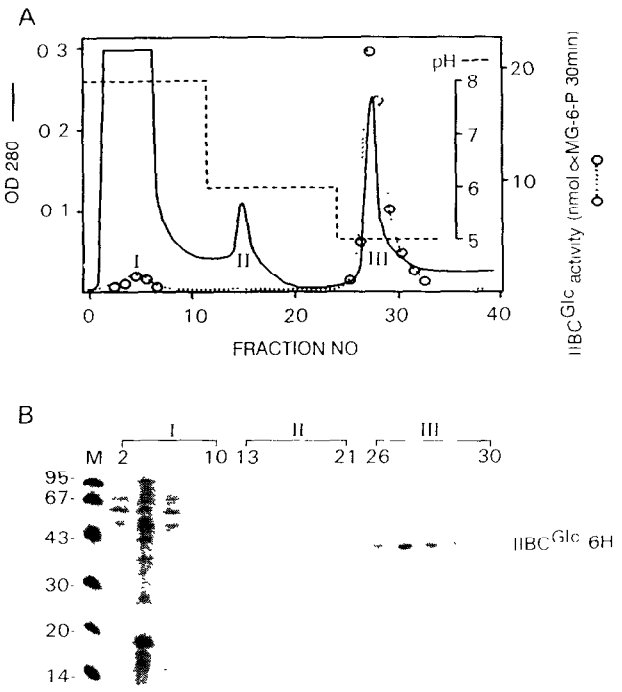

Fig. 1 Ni-chelate affinity chromatography of $IIBC^{Glc}$-6H. (a) Elution diagram 0.5 μl aliquots were assayed for phosphotransferase activity. (b) Coomassie blue stained polyacrylamide gel of NTA fractions. Every second fraction of peak I and peak II, and every fraction of peak III containing $IIBC^{Glc}$-6H is shown. M = molecular weight standards.

Yield and the specific activity of $IIBC^{Glc}$ obtained by Ni-NTA chromatography and by isolelectric focusing are comparable [8]. $IIBC^{Glc}$-6H is efficiently delipidated during Ni-NTA chromatography and gel filtration. The phosphate to protein mole ratio is 4.0 after Ni-NTA chromatography and less than 0.1 after gel filtration.

To our knowledge $IIBC^{Glc}$ is the first transmembrane protein to be purified by Ni chelate affinity chromatog-

Table I

Activity of $IIBC^{Glc}$-6H during purification

	Protein (mg)	Activity (μmol/30 min)	Spec. act. (μmol/30 min/mg)	Yield (%)	P_i (mol/mol)
Membranes	93.5	290	3.1	100	
TX-100 extract	47.3	246	5.2	85	
Ni-NTA	1.5	221	147.3	76	4.0 ± 0.1
FPLC	1.3	201	155.2	69	< 0.1

$IIBC^{Glc}$-6H was purified from 5 g wet weight of *E coli* ZSC112L transformed with plasmid pQEGH12 PEP:glucose phosphotransferase activity of $IIBC^{Glc}$-6H was assayed in the presence of purified enzyme I, HPr, IIA^{Glc} and phosphatidyl glycerol. $IIBC^{Glc}$-6H dependent formation of methyl α-D-glucopyranoside 6-phosphate was measured.

raphy. The high affinity of $IIBC^{Glc}$-6H indicates that the carboxy-terminal end of the hydrophilic IIB domain is accessible from the surface of the protein. Surface exposure of the carboxy-terminus is predicted by hydropathy analysis [1] and has been confirmed by the high activity of the fusion protein consisisting of $IIBC^{Glc}$ and the cytoplasmic subunit IIA^{Glc} [14]. The importance of surface exposure is stressed by the following observations: of three derivatives of the mannose transporter with tags at the N-terminus and the C-terminus of the IID^{Man} subunit and at the N-terminus of the IIC^{Man} subunit, all were fully active but only one with the tag at the most hydrophilic end (according to hydropathy analysis [15]) could successfully be purified (F. Huber and B. Erni, unpublished results). It appears that affinity tags too close to a transmembrane region might become sequestered in the detergent micelle and therefore become inaccessible to the Ni ions on the resin. This might be the reason for why purification of the lactose carrier (LacY), tagged with histidines in different internal and terminal positions, apparently did not meet with success [16].

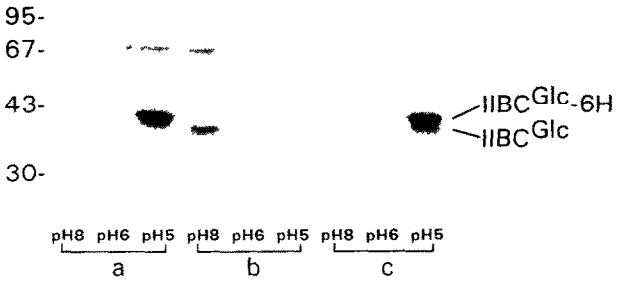

Fig. 2. Subunit exchange between $IIBC^{Glc}$ dimers. Purified $IIBC^{Glc}$-6H and $IIBC^{Glc}$ were mixed in a 4:1 ratio, separated on Ni-NTA agarose and analysed by polyacrylamide gel electrophoresis. (a) $IIBC^{Glc}$-6H binds at pH 8 and elutes at pH 5. (b) $IIBC^{Glc}$ (wild-type) does not bind at pH 8. (c) Heterodimers between $IIBC^{Glc}$-6H and $IIBC^{Glc}$ bind at pH 8 and elute at pH 5. Only a small amount of $IIBC^{Glc}$ (homodimers) does not bind at pH 8

3.2. *Other application of Ni-NTA in experiments with $IIBC^{Glc}$-6H*

Experiments like crystallization, reconstitution, and optical spectroscopy sometimes require an exchange of detergents. Binding of the tagged protein to Ni-NTA should facilitate this operation. $IIBC^{Glc}$-6H bound to Ni-NTA could be washed with detergent free buffer (five column volumes). After reequilibration with detergent (two column volumes), 70% of the bound activity could be recovered. It appears that resin bound $IIBC^{Glc}$ in contrast to the soluble protein cannot aggregate and does not denature after removal of detergent.

Ni-NTA binding appears a promising tool for the study of protein–protein interactions like subunit exchange in protein oligomers. When a mixture of $IIBC^{Glc}$ (wild-type) and $IIBC^{Glc}$-6H is added to the Ni-NTA resin, wildtype protein is retained together with $IIBC^{Glc}$-6H. Pure $IIBC^{Glc}$ without an affinity tag does not bind (Fig. 2). The subunit exchange between oligomers indicates that detergent solubilized $IIBC^{Glc}$ forms complexes which are kinetically labile at pH 8 but thermodynamically stable at pH between 6 and 8. $IIBC^{Glc}$ dimers have previously been shown by glutaraldehyde crosslinking, equilibrium centrifugation and velocity sedimentation [8,17]. The Ni-NTA procedure will be used to study the interaction of $IIBC^{Glc}$ with the IIA^{Glc} subunit and in particular the formation of heterodimers between subunits of transporters of different sugar specificity, e.g. between $IIBC^{Glc}$ and $IIABC^{GlcNAc}$. Intergenic complementation between these transporters has been shown [18–20] but the structural and functional basis for this effect is not yet clear. It will be interesting to see whether oligomerization is important for the phosphoryltransfer between the two phosphorylation sites on different subunits, for the vectorial translocation of the substrate or for both.

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