Expression of eukaryotic plasma membrane transporter HUP1 from *Chlorella kessleri* in *Escherichia coli*

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Abstract

To study the effect of sterols on the activity of the eukaryotic plasma membrane transporter, the hexose-proton symporter HUP1 from the unicellular alga *Chlorella kessleri* was expressed in *Escherichia coli*, a prokaryotic microorganism containing virtually no sterols. Under certain conditions, the recombinant protein was partially active in this prokaryotic organism. The heterologously produced HUP1p was purified from membrane fractions of *E. coli* and reconstituted in an in vitro system. The presence of ergosterol during solubilization, purification and reconstitution resulted in an increased activity of the reconstituted protein. Its activity, however, was 5–6 times lower as compared to the activity of HUP1p produced in *Saccharomyces cerevisiae* membranes and solubilized, purified, and reconstituted under the same conditions as above. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The primary function of lipids is to form a permeability barrier around cells and organelles by forming a phospholipid bilayer. The reason for the occurrence of a large number of different lipids in biological membranes is not completely understood, since a single bilayer-forming lipid would be sufficient for the membrane to form a permeability barrier and a matrix in which the proteins are embedded. Yet, a great variety of lipids differing in their polar head and/or the length and/or saturation of the hydrophobic chains are encountered in the membranes. In addition, in eukaryotic cells, membrane lipid composition is enriched by a number of different sterols (for review see [1,2]). Numerous data document that sterols alter the physico-chemical properties of biological membranes. They determine to a large extent the rigidity of the plasma membrane, which in turn may affect the lateral movement and the activity of membrane proteins. In addition to this largely non-specific effect, sterols have been shown to have a specific role in the function of eukaryotic membrane proteins [3–5].
Reconstitution experiments can contribute significantly to the elucidation of the role of sterols in the activity of membrane-bound proteins. Purification of a single membrane protein and its reinsertion into liposomes with defined lipid composition represents a simple system in which the lipid effects on the activities of membrane transporters, for example, can be directly measured. One should be aware, however, that some lipid molecules, which are probably the most important for the protein function, may remain tightly bound to the protein during solubilization under the mild conditions usually employed.

The HUP1 protein used as a model in this study is a hexose uptake protein from *Chlorella kessleri* [6]. Overexpression of the HUP1 protein in *Schizosaccharomyces pombe* [7] and *Saccharomyces cerevisiae* [8] lacking their inherent glucose transporters yielded a fully active permease with kinetic characteristics identical to those of *Chlorella* cells [9]. This suggests that the lipid composition of the membranes of these three microorganisms is similar or the putative differences are not decisive for HUP1p activity modulation. HUP1p has been purified and functionally reconstituted in proteoliposomes from crude *Escherichia coli* phospholipid [10]. Though no external sterols were included in the system, under the mild solubilization and purification conditions used, the HUP1 protein could have retained potentially tightly bound sterol molecules from its original membrane. To eliminate this possibility and to contribute to the elucidation of sterol effects on eukaryotic membrane-associated processes, we produced the eukaryotic transport protein HUP1 in *E. coli*, a prokaryotic microorganism containing virtually no sterols [11].

2. Materials and methods

2.1. Materials

*E. coli* L-α-phosphatidyl-ethanolamine type IX (P6398), ergosterol and anti-mouse IgG peroxidase conjugate were from Sigma. d-[U-¹⁴C]Glucose (295 mCi mmol⁻¹), streptavidin-horseradish peroxidase conjugate, and the ECL Western blot detection kit were from Amersham Buchler (Braunschweig, Germany). Octyl-β-glucoside was from Calbiochem (La Jolla, CA, USA); β-d-thiogalactopyranoside (IPTG) from AppliChem (Darmstadt, Germany). Immobilized monomeric avidin resin was obtained from Pierce (Rockford, IL, USA).

2.2. Organisms, growth and induction conditions

*E. coli* DH5α [12] was employed for all cloning steps. Two strains of *E. coli* were used for the expression of the recombinant protein: *E. coli* JWL191 ptsI191 glk⁻¹ lacking glucose transport (kindly supplied by Prof. J. Lengeler, Osnabrück) was originally used in this study because it was assumed to allow easy detection of functional expression of the heterologous hexose transporter. The strain was transformed by the construct pT7-HUP1 (see Section 2.3) concomitantly with pGP1-2 containing the gene for T7 RNA polymerase under the control of the λp₁ promoter that is repressed by a temperature-sensitive repressor [13]. It was then grown at 30°C in LB medium supplemented with ampicillin (100 μg ml⁻¹) and kanamycin (30 μg ml⁻¹). At OD₅₇₈ 0.4 the cells were transferred to 42°C for 30 min. After this period they were placed at 37°C. Since, however, the strain grew very poorly, and the heterologous expression resulted in growth cessation within 30 min, the more viable strain *E. coli* BL21 (DE3) LysS [14] was employed. This strain contains a chromosomal copy of the gene for T7 RNA polymerase under the control of the IPTG-inducible promoter lacUV5. Another advantage is that it carries a plasmid pLysS encoding lysozyme, which permits easier and more gentle breaking of the cells for membrane preparation. *E. coli* BL21 (DE3) LysS was transformed with the construct pT7-HUP1 and grown at 37°C in LB medium supplemented with ampicillin (100 μg ml⁻¹) and chloramphenicol (34 μg ml⁻¹). At OD₅₇₈ 0.5–0.6, expression of HUP1 was induced by addition of 1 mM IPTG.

2.3. Construction of translational expression vector pT7-7 HUP1-Bio-His6

HUP1-Bio-His6 cDNA in pUC18 constructed by Caspari et al. [10] (HUP1 tagged by biotinylation domain and six-histidine tail for the purpose of HUP1p affinity column purification) was cloned...
into the translational vector pT7-7 (Amp.) [15] containing a T7 promoter, a strong ribosome binding site (rbs) and starting ATG as a part of NdeI. For the sake of cloning in frame, an incomplete HUP1-Bio-His6 DNA shortened by 21 nucleotides at the N-terminus was prepared by partial digestion of HUP1-Bio-His6 cDNA in pUC18 with AvaI (cuts also at the XhoI site, an internal cleavage site in the construct) and BamHI. The 2.1-kb fragment was isolated (DNA fragment a). The translational vector pT7-7 was opened with NdeI and BamHI in the polycloning site and isolated (DNA fragment b). Oligonucleotides were designed to complete the N-terminus of HUP1-Bio-His6 DNA, creating the starting ATG as part of NdeI (5'-TATGGCCGCGGTG-GTGTAGTTGTTGTC-3' P) and a cohesive end for AvaI (5'-CCGAGACAACACTACACCCCGC-CGGCCA-3' (DNA fragment c). The three DNA fragments (a, b, c) were ligated in one mixture. For the sake of simplicity, the resulting construct is referred to as pT7-HUP1.

2.4. Identification of HUP1p expressed in E. coli

The recombinant protein was identified in whole cells, membrane fractions, solubilizates, eluates or in reconstituted vesicles after dissociating the samples in sample buffer [16] at 37°C for 10 min. SDS-PAGE was performed on 10% acrylamide gels using Bio-Rad Mini-Protein apparatus. HUP1 protein was identified after Western blotting either by specific monoclonal antibody (MAb 416b8, I. Robl, unpublished results) or by streptavidin-horseradish peroxidase conjugate (via its tagged biotinylation domain).

2.5. Isolation of membranes from E. coli and S. cerevisiae

E. coli 24 membranes were prepared essentially as described in [16]. Yeast crude membrane fraction was prepared as described in [10].

2.6. Solubilization of E. coli membranes, purification and reconstitution of HUP1 protein

The membranes were solubilized and the protein purified on immobilized monomeric avidin resin as described [10]. The protein was reconstituted into proteoliposomes as in [10]. The internal volume of the reconstituted proteoliposomes was estimated as in [13] and was 0.98 and 1.12 μl mg−1 phospholipid for ergosterol-free and ergosterol-containing vesicles, respectively.

2.7. Measurement of membrane potential and transport assay

The proteoliposomes were energized by addition of the electron-donor system of cytochrome c oxidase (20 mM ascorbate, 20 μM cytochrome c, 200 μM TMPD). Measurement of membrane potential and transport assay were performed as described [17].

2.8. Miscellaneous

Protein concentration was measured according to Bradford [18] with BSA as a standard.

3. Results and discussion

3.1. Expression of HUP1 in E. coli JWL191 ptsI191 glk1 and BL 21 (DE3) LysS

The HUP1p expression in the mutant strain E. coli JWL191 ptsI191 glk1 lacking glucose transport was induced by heat shock inducible T7 RNA polymerase carried on pGP1-2 [14]. As documented in Fig. 1A, within 30 min after transferring the transformed cells to 42°C, a band corresponding to HUP1 protein could be detected in whole cell extract. Its apparent molecular mass corresponded to 58 kDa, similar to that reported for the yeast-produced protein [10]. Fractionation analysis of the overexpressing cells showed that the biotinylated protein was associated with the membrane fraction (data not shown). We therefore examined whether the eukaryotic plasma membrane protein from unicellular algae was incorporated functionally into the membranes of E. coli. In vivo transport experiments, however, showed no restoration of glucose transport in the cells expressing a considerable amount of HUP1p. The mutant cells containing the two plasmids (pT7-HUP1 and pGP1-2) were seriously impaired in their growth.

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Before the heat shock induction, they doubled in 1.5 h (as compared to 20–30 min for the wild-type strain), and ceased growing at OD578 ∼ 0.6 after induction. For this reason a more viable strain, E. coli BL 21 (DE3) LysS, was employed. The transformed cells (bearing two plasmids, pLysS and pT7-HUP1) grew with a generation time of about 30 min, which corresponded to that found in control strain transformed by empty pT7-7. The induced cell culture grew with the same generation time until OD578 ∼ 1.6. The recombinant HUP1p was detected in whole cell extract by both a monoclonal antibody and streptavidin-peroxidase conjugate before and at intervals after the IPTG induction (Fig. 1C, B, respectively). The latter more sensitive detection revealed a small amount of the protein produced in cells at the beginning of exponential growth even before IPTG induction, indicating that the system was somewhat leaky. The elevated production of HUP1p was observed as early as 10 min after the induction and it was increasing for at least 2 h when the cells continued growing.

Cells of E. coli BL21 (DE3) LysS possess an inherent glucose transport mediated by vectorial phosphorylation by phosphoenolpyruvate, the phosphotransferase system (PTS) [19]. The activity of PTS would interfere with the estimation of glucose uptake putatively mediated by the recombinant protein. The initial rates of glucose uptake were therefore compared in the transformed cells before and after IPTG induction (Fig. 2).

Fig. 1. A: Western blot analysis of HUP1p expression in whole cell extracts of E. coli JW191 before (lane 1) and after heat shock (lane 2), and subsequent 30 and 60 min cultivation at 37°C (lanes 3, 4). Amounts of cell extracts corresponding to 0.1 OD578 units were resolved by SDS-PAGE and HUP1p was identified by streptavidin-horseradish peroxidase conjugate. B: Western blot analysis of HUP1p expression in E. coli BL21 (DE3) LysS before (lane 1) and 30, 60, 120 and 180 min after induction by IPTG (lanes 2, 3, 4, 5, respectively). Amounts of cell extracts corresponding to 0.1 OD578 units were resolved by SDS-PAGE and HUP1p was identified by streptavidin-horseradish peroxidase conjugate. C: Western blot analysis of HUP1p expression in E. coli BL21 (DE3) LysS before (lane 1) and 30, 60 and 120 min after induction by IPTG (lanes 2, 3, 4, respectively). The amounts of cell extracts corresponding to 0.1 OD578 units were resolved by SDS-PAGE and HUP1p was identified by specific monoclonal antibody MAb 416b8. HUP1p is indicated by the arrow. Representative of three independent experiments.
Even though the recombinant protein HUP1 was located in the membrane fraction (see above), comparison of induced versus uninduced cells showed that only a relatively small increase in glucose uptake could be attributed to the *E. coli*-produced HUP1p. This could be explained as being due either to a disturbance in cell function and viability and/or to impaired activity of the recombinant protein. We tried to profit from the leakiness of the IPTG-inducible expressing system and produce only a moderate amount of HUP1p in the cell. Indeed, a reasonable increase in the initial rate of glucose uptake was found in the uninduced transformed cells as compared to the induced cells in the same stage of growth which produced a considerable (possibly interfering) amount of HUP1p (Fig. 2). To check whether the *E. coli*-inherent PTS activity differed in relation to the growth phase, the initial glucose uptake rates were compared in the uninduced HUP1 bearing cells (producing a moderate amount of the HUP1p) and in the cells transformed with the pT7-7 empty plasmid. As shown in Fig. 3, the uptake rate differences varied with growth phase.

The data above show that it is possible to overproduce a H+/glucose symporter from a unicellular alga in membranes of *E. coli* in quite large amounts. In the mutant strain, *E. coli* JWL191 ptsI191 glk1, the toxicity of this overproduction was demonstrated very early on, while in the other strain (*E. coli* BL21 (DE3) LysS) the cessation of growth set in only after a period of 2 h. The large amount of heterologous protein produced in induced cells within this period, however, obviously interfered with cell physiology and was therefore not paralleled by an increased transport activity. Only when a moderate amount of the recombinant protein was produced in the uninduced cells, due to the leakiness of the system, were we able to detect glucose uptake attributable to the heterologous protein.

In the next step we tested whether differences in lipid composition between eukaryotic and prokaryotic membranes were responsible for the rather low activity of the HUP1 protein.

Fig. 2. Glucose accumulation in *E. coli* BL21 (DE3) LysS expressing HUP1p. The initial glucose uptake was measured in one part of uninduced cell culture at time 0 (OD$_{578}$ = 0.5) (□) and after 60 min (OD$_{578}$ = 2.1) (▲). The other part of the cell culture was induced by IPTG and the glucose uptake was measured after 30 (■) and 60 (●) min. The concentration of 14C-labeled glucose was 80 μM. Bottom: Lanes 1, 2, 3, and 4 represent the amount of HUP1p identified by streptavidin-horseradish peroxidase conjugate in cells tested for glucose uptake and correspond to curves denoted □, ■, ● and ▲, respectively. Representative of two independent experiments.

Fig. 3. Initial uptake rate of glucose in uninduced *E. coli* BL21 (DE3) LysS bearing either empty vector pT7-7 (empty columns) or pT7-HUP1 (filled columns) as dependent on growth phase. Mean of two independent experiments.
3.2. Reconstitution of purified HUP1 into proteoliposomes

The phospholipid composition of the *E. coli* membrane differs from those of *Chlorella* and yeast in which HUP1p functions efficiently. Phosphatidylethanolamine (PE) (70–80%) is the main phospholipid of the *E. coli* inner membrane; phosphatidylglycerol (10–20%) and cardiolipin (1–10%) are the other two main components [20]. The yeast plasma membrane (and probably also that of *Chlorella*; data not available) contains, in addition to PE, a considerable amount of phosphatidylcholine; phosphatidylinositol and phosphatidylserine [21]. In addition, in contrast to *Chlorella* and yeast cells, *E. coli* does not contain sterols. Therefore, we focused on the effect of sterols on HUP1p activity. In the first approach, ergosterol was used as a test sterol since the HUP1p is fully active in the yeast plasma membrane [8] which contains exclusively ergosterol, and ergosterol is also found in the *Chlorella* cells [22].

The recombinant protein was solubilized from membrane fractions of the two overproducing *E. coli* strains tested above, and purified in the absence...
or in the presence of 10% ergosterol. The purified protein was then incorporated into proteoliposomes together with cytochrome c oxidase as a proton motive force (pmf) generator. After energization, the vesicles were tested for the transport activity of the HUP1p. As documented in Fig. 4A,B, glucose accumulation slightly above the equilibrium was observed in vesicles containing HUP1p produced in both types of cells. However, when the protein was solubilized, purified and reconstituted in the presence of ergosterol, the glucose accumulation increased 2–3-fold as compared with ergosterol-free vesicles. Since the glucose accumulation depends on the actual amount of HUP1p incorporated in the vesicles as well as on the magnitude of the pmf formed in the system, both parameters were checked for each transport experiment. Membrane potential is the main pmf component driving glucose accumulation [17]. Its magnitude was used as a measure of energization of the system. The amounts of protein incorporated for each individual uptake curve are shown in Fig. 4, bottom. Since the driving force for accumulation as well as the amount of the protein in ergosterol-free and ergosterol-containing vesicles were comparable, the increased uptake of glucose could be attributed to the presence of ergosterol. As a control experiment, the activity of HUP1 protein produced in yeast was tested. Irrespective of the addition of external ergosterol, this HUP1p incorporated into the proteoliposomes caused a 5–6-fold higher accumulation of glucose as compared to the protein produced in E. coli (Fig. 4B).

In general, sterols can affect the activity of plasma membrane proteins via changes of the physico-chemical state of the plasma membrane. If the presence of ergosterol in our reconstitution system caused significant changes in the membrane properties, the activity of pmf generator (cytochrome c oxidase) and proton permeability might have been affected, as well. The fact that the course of the formation as well as the magnitude of the membrane potential was the same in both ergosterol-containing and ergosterol-free vesicles suggests that this was not the case. A more specific effect of the sterol could be envisaged, such as faulty incorporation of membrane spanning helices of protein from sterol-containing membrane into the thinner sterol-free membrane. This could lead to an incorrect folding of the protein in the membrane [23]. When solubilized and allowed to refold in a sterol-containing membrane, some of the protein molecules might fold in an active form. In several reconstitution experiments employing mammalian and higher plant plasma membrane proteins, even more specific roles of sterols have been documented [24–27]. In vitro studies of the effect of nystatin on the activity of arginine transporter in yeast suggested a close interaction of the permease with ergosterol molecules [28,29].

The results obtained in this study document that the addition of ergosterol to sterol-devoid eukaryotic plasma membrane transporter can lead to partial recovery of its activity in a reconstituted system. The fact that the same protein, when produced in yeast, is more active after its reconstitution might be due to several reasons. (i) The ergosterol may assist in functional folding of the transporter in vivo. (ii) Possibly tightly bound molecules of the sterol preserve the active conformation of the transporter during purification from yeast while exogenously added sterol is only partially efficient in restoring the native conformation of sterol-free (E. coli-produced) transporter. (iii) Besides the misfolding and/or more specific effects of ergosterol, the lack of machinery carrying out possible posttranslational modifications (e.g., phosphorylation or glycosylation) in the bacterial host might be responsible for the poor activity of the recombinant protein. Neither phosphorylation nor glycosylation, however, has experimentally been demonstrated for HUP1p (unpublished data). (iv) Of course, some other factors cannot be excluded.

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References


