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Production of highly phosphorylated glycopolymers by expression of R1 in *Escherichia coli*

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Abstract

The possible involvement of the starch bound R1 protein from potato (*Solanum tuberosum* L.) in the phosphorylation of starch was investigated by functional expression and characterisation of R1 in *Escherichia coli*. By expression of R1 in *E. coli* it is shown that it is possible to produce glycopolymers, e.g., glycogen, with an increased degree of phosphate substitution. The expression of R1 in *E. coli* resulted in a sixfold increase in glycogen bound phosphate and in an increased accumulation of glycogen leading to a glycogen excess (gex) phenotype. There was an overall shift in the unit-chain length of the isolated glycogen towards smaller degrees of polymerisation. The pleiotropic effects on the glycogen biosynthetic and amylolytic enzyme activities was investigated and showed an increase in ADPglucose pyrophosphorylase activity, as well as a decrease in exo-amylolytic activity. These results are discussed in relation to starch phosphorylation and a possible role of R1 in this respect. © 2002 Published by Elsevier Science Ltd.

Keywords: R1 starch protein; Glycogen; Phosphorylation; Bacterial recombinant proteins; Pyrophosphorylase; Potato

1. Introduction

Most bacteria store glucose in glycogen bodies, consisting of water-soluble α-(1→4)-linked polysaccharides with up to 10% α-(1→6)-branch points (reviewed in Manners[1]), whereas plants store glucose in the form of starch granules. In contrast to glycogen, starch consists of amylose that is essentially linear, and of partially crystalline amylopectin, that contains up to 5% α-(1→6)-branch points (reviewed by Smith et al.[2]).

Amylopectin from potato tuber starch contains small amounts of covalently bound phosphate, i.e., one glucose unit out of 200–300 is phosphorylated.[3] The phosphate groups are bound as monoesters at C-6 (approx. 70%) and at C-3 (approx. 30%) positions of the glucose residues. In addition, a small fraction (1%) may be linked to C-2.[3,4] Phosphate is also present in glycogen although to a much less extent, i.e., one glucose unit out of 1600 is phosphorylated.[5] A high degree of phosphate substitution in starch results in starch gels with high viscosity and stable starch pastes.[6,7]

In contrast to starch, phosphate in glycogen is bound as monoesters at the C-6 position or as glucosyl-1,6-glucose phosphodiesters in glycogen in a ratio of 40:60, respectively.[5,8,9] Due to the fact that both starch and glycogen contain monoesterified phosphate, *Escherichia coli* constitutes an ideal model organism when studying the phosphorylation of starch, since the biosynthetic pathways leading to starch or glycogen are comparable. In both pathways ADPglucose pyrophosphorylase (EC 2.7.7.27) converts Glc-1P into ADPglucose, the substrate for starch and glycogen synthases (EC 2.4.1.21).[2,10] An additional common step in starch and

Abbreviations: AGPase, ADPglucose pyrophosphorylase; DP, degree of polymerisation; *E. coli*, *Escherichia coli*; GBE, Glycogen branching enzyme; Glc-1P, glucose-1-phosphate; Glc-6P, glucose-6-phosphate; HPAEC, high-pressure anion-exchange chromatography.

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glycogen biosynthesis is the introduction of α-(1→6)-branch points which are catalysed by branching enzyme (EC 2.4.1.18).[11,12] The mechanism by which phosphate is incorporated into starch remains elusive, but since both starch and glycogen contains monoesterified phosphate, the precursors and enzyme activities responsible for the phosphorylation process must be present in both plants and bacteria. In mammalian cells, a UDPglucose:glycogen glucose 1-phosphotransferase has been recognised as being responsible for the positioning of glucose 1-phosphate on C-6 of glucose residues in glycogen, thereby forming the phosphodiester as described above.[8]

Recently a starch bound protein, designated R1, was isolated from potato.[13] The function of this protein is currently unknown, but findings by Lorberth et al.[13] suggest that R1 is involved in the phosphorylation of starch, since antisense inhibition of the gene encoding R1 results in a 90% reduction in starch bound phosphate. It has also been shown that by expressing R1 in *E. coli*, it was possible to more than double the phosphorylation of glycogen. Lorberth et al.[13] also speculated that R1 may be involved in the degradation of starch since starch accumulates in the leaves of R1 antisense plants after prolonged periods in the dark.

In this study we describe the cloning and effects of expressing a new isoform of R1 in *E. coli* on the glycogen structure and phosphate content, as well as the effects on glycogen biosynthetic and amylolytic enzyme activities. Our results leads us to hypothesise that R1 is somehow involved in the phosphorylation and regulation of the degradation of glycogen leading to a glycogen excess phenotype in the *E. coli* strain over-expressing R1 and a changed structure of the glycogen.

2. Results

Cloning of a full-length R1 cDNA clone.—Cloning of a full-length R1 cDNA clone resulted in isolation of a clone with a deduced amino acid sequence different from that already published.[13] In Fig. 1 is shown an alignment of the two deduced amino acid. As can be seen from Fig. 1, the sequence isolated in this study differ with 24 amino acid, i.e., resulting in 98% similarity, compared to that reported found in (*Solanum tubersoum* L. cv. 'Berolina').[13] This suggests the existence of a second isoform of R1 in potato, or alternatively, a second allo-form of R1. The amino acid differences might also reflect simple cultivar differences, since the two clones have been isolated from different cultivars. The existence of more than one R1 isoform in the same plant specie is known from at least one other plant specie, i.e., *Arabidopsis thaliana*. A search in the Genbank sequence database reveals two R1 homologues, with accession number AF312027 and CAB79355 from *Arabidopsis*.

Expression of R1.—A high-expression level of the R1 protein was achieved (Fig. 2). The highest expression was observed when inducing the cell cultures at $OD_{600\ nm} = 1.0$ and subsequent growth of the cell cultures for 20 h. Induction at a lower $OD_{600\ nm}$ value and at a shorter induction period lead to poorer yields of recombinant protein. The R1-thioredoxin fusion protein migrated with an apparent molecular mass of approximately 170 kDa. This corresponds very well with the mass for R1, 157 kDa, combined with the mass of 12.7 kDa from the thioredoxin part of the polypeptide.[13]

Phenotype of E. coli *strain expressing R1.*—The *E. coli* strain expressing R1 was investigated microscopically after iodine staining in order to visualise the glycogen bodies inside the cells (Fig. 3). Moreover, time course quantitative measurements of glycogen accumulation in the vector control and the R1 producing *E. coli* strain, were performed. Whereas the production of proteins in the two *E. coli* strains is identical, the *E. coli* strain that expresses R1 stores up to 50% more glycogen (Fig. 4(B)). Furthermore, the *E. coli* cells expressing R1 typically store more than one glycogen body per cell (Fig. 3(B)), while wild-type and *E. coli* vector control cells were never observed to contain more than one glycogen body per cell.

Molecular structure of resulting glycogen.—The molecular structure of glycogen stored in the *E. coli* strain expressing R1 was compared with that accumulated in the vector control strain. Typical yields were 15 and 8 mg glycogen for the two strains, respectively, using 600 mL cell cultures. Whereas the degree of phosphorylation of glycogen isolated from the vector control strain was 0.9 nmol Glc-6P mg^{-1} glycogen, the content in the R1 expressing strain was sixfold higher, i.e., 5.2 nmol Glc-6P mg^{-1} glycogen (Table 1).

The chain-length distribution of the unit chains of the isolated glycogen from the two strains also differed as presented in Fig. 5. The glycogen from the *E. coli* strain expressing R1 had significantly shorter chains with a peak at DP 10 in contrast to DP 12 in the control strain. It is especially the higher fraction of short chains (DP 3–10) in the R1 strain that is responsible for the overall shift in chain-length distribution.

Effect on glycogen biosynthetic and amylolytic activities.—Since no enzymatic activity of R1 has been revealed yet, possible pleiotropic effects on the glycogen biosynthetic and amylolytic enzyme activities were investigated (Fig. 6).[13,14] The activity of the ADPglucose pyrophosphorylase was up-regulated approximately 60% in the R1 strain (Fig. 6(A)), whereas the glycogen synthase activity was unchanged. The activity of the glycogen branching enzyme measured, using both the phosphorylase *a* stimulation assay and the iodine-stain-

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    R1  MSNSLGNNLL YQGFLTSTVL EHKSRISPPC VGGNSLFQQQ VISKSPLSTE FRGNRLKVQK KKIPMGKNRA
t07050  MSNSLGNNLL YQGFLTSTVL EHKSRISPPC VGGNSLFQQQ VISKSPLSTE FRGNRLKVQK KKIPMEKKRA

    R1  FSSSPHAVLT TDTSSQLAEK FSLEGNIELQ VDVRPPTSGD VSFVDFQVTN GSDKLFLHWG AVKFGKETWS
t07050  FSSSPHAVLT TDTSSELAEK FSLGGNIELQ VDVRPPTSGD VSFVDFQVTN GSDKLFLHWG AVKFGKETWS

    R1  LPNDRPDGTK VYKNKALRTP FVKSGSNSIL RLEIRDTAIE AIEFLIYDEA HDKWIKNIGG NFHVKLSRKE
t07050  LPNDRPDGTK VYKNKALRTP FVKSGSNSIL RLEIRDTAIE AIEFLIYDEA HDKWIKNNGG NFRVKLSRKE

    R1  IRGPDVSVPE ELVQIQSYLR WERKGKQNYT PEKEKEEYEA ARTELQEEIA RGASIQDIRA RLTKTNDKSQ
t07050  IRGPDVSVPE ELVQIQSYLR WERKGKQNYP PEKEKEEYEA ARTVLQEEIA RGASIQDIRA RLTKTNDKSQ

    R1  SKEEPLHVTK SNIPDDLAQA QAYIRWEKAG KPNYPPEKQI EELEEARREL QLELEKGITL DELRKKITKG
t07050  SKEEPLHVTK SDIPDDLAQA QAYIRWEKAG KPNYPPEKQI EELEEARREL QLELEKGITL DELRKTITKG

    R1  EIETKVEKHL KRSSFAVERI QRKKRDFGQL INKYPSSPAV QVQKVLEEPA ALSKIKLYAK EKEEQIDDPI
t07050  EIKTKVEKHL KRSSFAVERI QRKKRDFGHL INKYTSSPAV QVQKVLEEPP ALSKIKLYAK EKEEQIDDPI

    R1  LNKKIFKVDD GELLVLVSKS SGKTKVHLAT DLNQPITLHW ALSKSPGEWM VPPSSILPPG SIILDKAAET
t07050  LNKKIFKVDD GELLVLVAKS SGKTKVHLAT DLNQPITLHW ALSKSPGEWM VPPSSILPPG SIILDKAAET

    R1  PFSASSSDGL TSKVQSLDIV IEDGNFVGMP FVLLSGEKWI KNQGSDFYVD FSAASKSALK AAGDGSGTAK
t07050  PFSASSSDGL TSKVQSLDIV IEDGNFVGMP FVLLSGEKWI KNQGSDFYVG FSAASKLALK AAGDGSGTAK

    R1  SLLDKIADME SEAQKSFMHR FNIAADLMED ATSAGELGFA GILVWMRFMA TRQLIWNKNY NVKPREISKA
t07050  SLLDKIADME SEAQKSFMHR FNIAADLIED ATSAGELGFA GILVWMRFMA TRQLIWNKNY NVKPREISKA

    R1  QDRLTDLLQN AFTSHPQYRE ILRMIMSTVG RGGEGDVGQR IRDEILVIQR KNDCKGGMME EWHQKLHNNT
t07050  QDRLTDLLQN AFTSHPQYRE ILRMIMSTVG RGGEGDVGQR IRDEILVIQR NNDCKGGMMQ EWHQKLHNNT

    R1  SPDDVVICQA LIDYIKSDFD LGVYWKTLNE NGITKERLLS YDRAIHSEPN FRGDQKGGLL RDLGHYMKTL
t07050  SPDDVVICQA LIDYIKSDFD LGVYWKTLNE NGITKERLLS YDRAIHSEPN FRGDQKGGLL RDLGHYMRTL

    R1  KAVHSGADLE SAIANCMGYK TEGEGFMVGV QINPVSGLPS GFQDLLHFVL DHVEDKNVET LLERLLEARE
t07050  KAVHSGADLE SAIANCMGYK TEGEGFMVGV QINPVSGLPS GFQDLLHFVL DHVEDKNVET LLERLLEARE

    R1  ELRPLLLKPN NRLKDLLFLD IALDSTVRTA VERGYEELNN ANPEKIMYFI SLVLENLALS VDDNEDLVYC
t07050  ELRPLLLKPN NRLKDLLFLD IALDSTVRTA VERGYEELNN ANPEKIMYFI SLVLENLALS VDDNEDLVYC

    R1  LKGWNQALSM SNGGDNHWAL FAKAVLDRTR LALASKAEWY HHLLQPSAEY LGSILGVDQW ALNIFTEEII
t07050  LKGWNQALSM SNGGDNHWAL FAKAVLDRTR LALASKAEWY HHLLQPSAEY LGSILGVDQW ALNIFTEEII

    R1  RAGSAASLSS LLNRLDPVLR KTANLGSWQI ISPVEAVGYV VVVDELLSVQ NEIYEKPTIL VAKSVKGEEE
t07050  RAGSAASLSS LLNRLDPVLR KTANLGSWQI ISPVEAVGYV VVVDELLSVQ NEIYEKPTIL VAKSVKGEEE

    R1  IPDGAVALIT PDMPDVLSHV SVRARNGKVC FATCFDPNIL ADLQAKEGRI LLLKPTPSDI IYSEVNEIEL
t07050  IPDGAVALIT PDMPDVLSHV SVRARNGKVC FATCFDPNIL ADLQAKEGRI LLLKPTPSDI IYSEVNEIEL

    R1  QSSSNLVEVE TSATLRLVKK QFGGCYAISA DEFTSEMVGA KSRNIAYLKG KVPSSVGIPT SVALPFGVFE
t07050  QSSSNLVEAE TSATLRLVKK QFGGCYAISA DEFTSEMVGA KSRNIAYLKG KVPSSVGIPT SVALPFGVFE

    R1  KVLSDDINQG VAKELQILTK KLSEGDFSAL GEIRTTVLDL SAPAQLVKEL KEKMQGSGMP WPGDEVPKRW
t07050  KVLSDDINQG VAKELQILMK KLSEGDFSAL GEIRTTVLDL SAPAQLVKEL KEKMQGSGMP WPGDEGPKRW

    R1  EQAWMAIKKV WASKWNERAY FSTRKVKLDH DYLCMAVLVQ EIINADYAFV IHTTNPSSGD DSEIYAEVVR
t07050  EQAWMAIKKV WASKWNERAY FSTRKVKLDH DYLCMAVLVQ EIINADYAFV IHTTNPSSGD DSEIYAEVVR

    R1  GLGETLVGAY PGRALSFICK KKDLNSPQVL GYPSKPIGLF IKRSIIFRSD SNGEDLEGYA GAGLYDSVPM
t07050  GLGETLVGAY PGRALSFICK KKDLNSPQVL GYPSKPIGLF IKRSIIFRSD SNGEDLEGYA GAGLYDSVPM

    R1  DEEEKVVIDY SSDPLITDGN FRQTILSNIA RAGHAIEELY GSPQDIEGVV RDGKIYVVQT RPQM
t07050  DEEEKVVIDY SSDPLITDGN FRQTILSNIA RAGHAIEELY GSPQDIEGVV RDGKIYVVQT RPQM
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Fig. 1. Amino acid alignment of the R1 clone isolated in this study (designated R1) and that reported earlier, designated t07050 (Genbank accession number).[13] The amino acid differences between the two sequences are marked in grey.

ing assay (Fig. 6(C and D)), showed no significant differences between the two strains. In contrast, the exo-amylase activity (Fig. 6(E)) was down regulated 55%, in the R1 strain as compared to the control strain.

3. Discussion

In this study, we demonstrate that the functional expression of R1 in *E. coli* affects glycogen metabolism

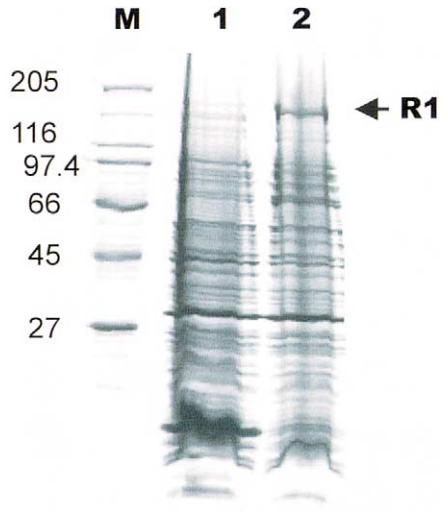

Fig. 2. Protein profiles of *E. coli* cells expressing R1 as analysed by SDS-PAGE. (Lane 1) *E. coli* vector control strain; (Lane 2) *E. coli* strain expressing R1. The molecular weights of marker proteins are indicated. Total protein from 100 μL cell culture is applied to each lane.

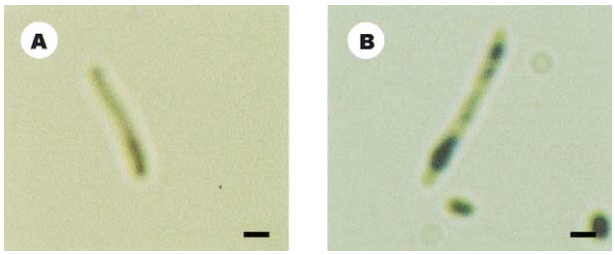

Fig. 3. The phenotype of *E. coli* expressing R1 as analysed by light microscopy. (A) Vector control strain; (B) *E. coli* strain expressing R1. Before visualisation, the cells were stained with an iodine solution (26 g KI/2.6 g I_2 in 100 mL 0.1 N HCl, diluted 1:1000 before use). The black bar represents 10 μm.

resulting in both quantitative and qualitative alterations of glycogen accumulation. In contrast to the situation in plants, where suppression of R1 results in a starch excess phenotype, an increased expression of R1 in *E. coli* results in a higher rate of accumulation of glycogen (Fig. 4), i.e., a glycogen excess (gex) phenotype with several glycogen bodies per cell. The glycogen accumulated in the R1 expressing *E. coli* strain shows a nearly sixfold increase in glycogen bound phosphate which is threefold more than reported.[13] Our results support the hypothesis that R1 is involved in the phosphorylation of starch although the mechanism remains elusive.[13,14] The glycogen phosphorylation is associated with a change in glycogen structure, as documented by an altered chain-length distribution (Fig. 5). This is the result of an increased DP 3–10 population, and a decrease in the DP 12–35 population in the R1 strain directing the total-chain population towards shorter DP.

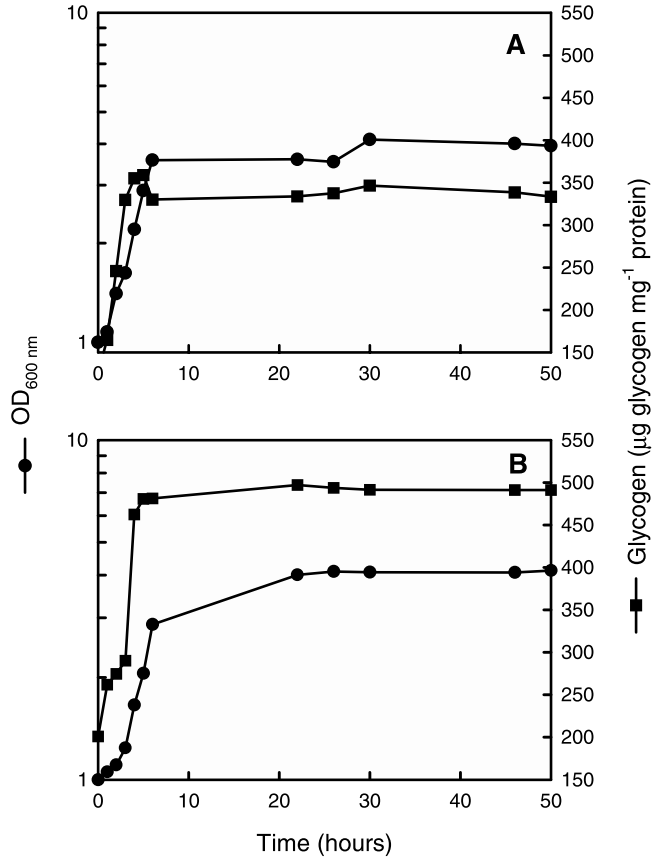

Fig. 4. Time course study of the glycogen accumulation in *E. coli* expressing R1. (A) *E. coli* vector control strain; (B) *E. coli* strain expressing R1. At each data point, glycogen and protein were extracted from identically sized aliquots of a continuously growing cell culture.

Table 1
Amount of glycogen bound phosphate in the *E. coli* strain expressing R1 as compared to a vector control strain

E. coli strain	Glycogen bound phosphate (nmol Glc-6P mg^{-1} glycogen)
Vector control	0.9 ± 0.2
+R1	5.2 ± 0.5

In an effort to delineate a possible biochemical basis for the observed phenotype and the structural changes of the glycogen, glycogen biosynthetic enzyme activities and amylolytic activities were measured (Fig. 6). The increased AGPase activity in the R1 strain can explain the observed increase in glycogen accumulation, since the reaction catalysed by AGPase is the first committed step in both glycogen and starch biosynthesis.[10,15,16] The increased glycogen accumulation is in agreement with the observations that showed that by increasing the substrate availability for starch synthesis by increasing the AGPase activity leads to a higher accumulation of starch in potato tubers.[17] The increase in glycogen

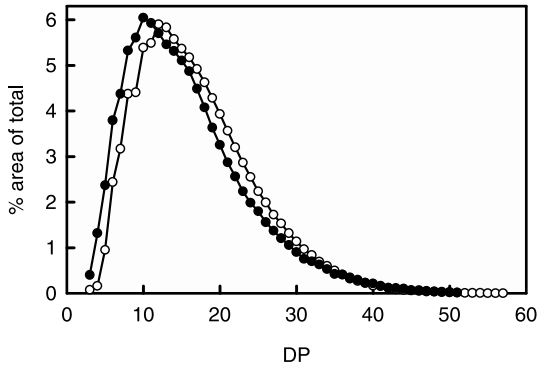

Fig. 5. Chain length distribution of glycogen side-chains as analysed by HPAEC. ○, Glycogen isolated from the *E. coli* vector strain. ●, Glycogen isolated from the *E. coli* strain expressing R1.

accumulation may also be explained by the fact the phosphate present in glycogen, as a results of the introduced R1 protein, activates the glycogen synthase as suggested by Lomako et al.,[9] leading to a higher rate of synthesis. Another explanation could also be that the C-6 bound phosphate in the glycogen simply hinders the mobilisation of glycogen by blocking the action of amylolytic enzymes, which has been shown in maltodextrins and starch.[18]

The observed down-regulation of exo-amylase activity in the R1 strain could also contribute to the observed gex phenotype as an effect of a reduced ability to re-mobilise glycogen. The altered chain-length distribution observed could be a direct effect of the reduction in amylolytic activity, which may limit the extent of 'trimming' of the glycogen molecule during de novo synthesis on assumption that amylases participate in the 'glucan trimming model'.[19] The down-regulation of the exo-amylase activity could be direct effects of R1 expression if R1 functions as a regulatory protein of amylases.

The effect on glycogen metabolism as a result of R1 over-expression in the *E. coli* cell demonstrates a profound interaction of R1 with *E. coli* primary metabolism. Its presence affects many enzyme activities with a multitude of consequences on the glycogen metabolism, as demonstrated by altered structure and increased deposition rates. The same situation has been shown for potato tubers where starch branching enzyme or starch synthases have been down regulated.[20,21] In these situations, tight relationships between starch phosphorylation and the degree of starch branching were demonstrated. For natural starches, the same relationships were found, i.e., high-starch phosphate contents are found in starches with long amylopectin unit chains.[22,23] However, in potato tubers with suppressed R1 content changes in the branching pattern of the starch could not be detected.[24] Furthermore, no correlation between R1 expression and the degree of starch phosphorylation in different botanical species has been found.[14] This would suggest that activities of SBEs or SS's normally determine the degree of phosphorylation and that the effects of R1 are not limiting in most plants.

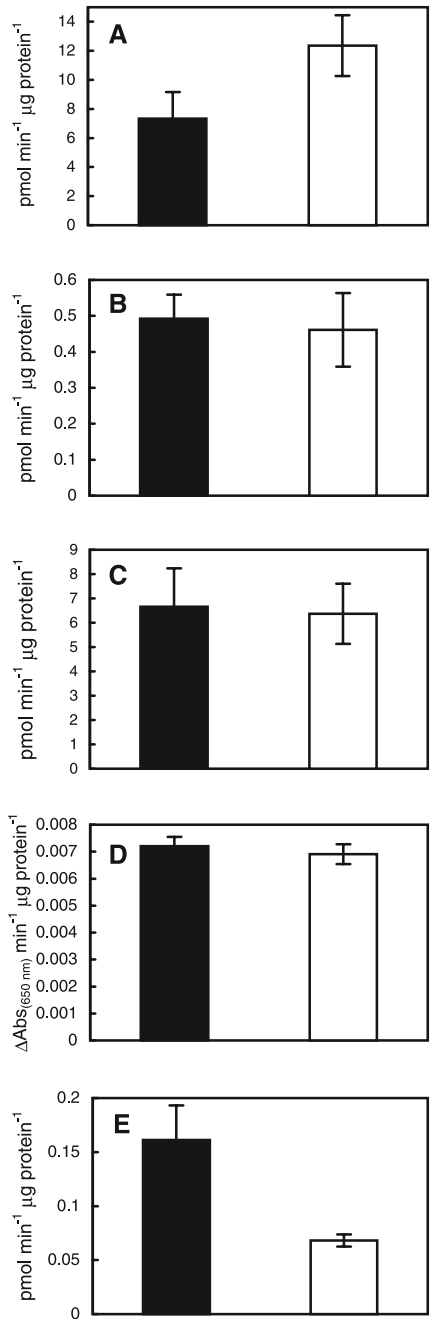

Fig. 6. The effect of expression of R1 in *E. coli* on the activities of glycogen biosynthetic and amylolytic enzymes. Black bar: enzyme activity in the *E. coli* vector control strain. White bar: enzyme activity in the *E. coli* strain expressing R1. (A) ADPglucose pyrophosphorylase; (B) glycogen synthase; (C) glycogen branching enzyme (phosphorylase *a* stimulation assay); (D) glycogen branching enzyme (iodine staining assay); (E) exo-amylase.

In contrast to plants, the effects of potato R1 on *E. coli* glycogen metabolism are, except with respect to phosphate content, not the same. Increased levels of R1 and glycogen bound phosphate result in shorter glycogen unit chains. Moreover, in potato leaves with down regulated R1, a starch-excess phenotype was observed, but in *E. coli* a glycogen-excess phenotype is the result of very high levels of R1 and of covalently bound phosphate.[13] This suggests that R1-mediated starch phosphorylation may have a regulatory role in the cell, and depending on the different metabolic situations in a plant cell and in an *E. coli* cell, the effects of its presence are diverse. It may be suggested that the primary effect of R1 is phosphorylation of storage α-glucan polymers since phosphorylation is positively correlated with R1 expression when R1 is expected to be limiting in both potato tubers and in *E. coli*, while the effects on α-glucan deposition rates are opposite in these two organisms. This indicates that the latter effects are pleiotropic, caused by the altered phosphate pools in the cells including phosphorylated intermediates, which function as signals for starch synthesis, e.g., AGPase and glycgen/starch synthase and mobilisation, e.g., amylases. A thorough analysis of metabolite levels in these organisms would shed light on these problems.

The finding of a possible second iso- or allo-form of R1 (Fig. 1) might reflect alternative expression pattern of the two, depending on either, the metabolic status in the cell or different tissue specificities, although not examined.

Due to the fact that *E. coli* contains the full enzymatic machinery for synthesising phosphorylated glycogen, as shown in this study, *E. coli* constitutes a powerful model organism for further studies of enzyme activities involved in the biosynthesis and phosphorylation of starch.

4. Experimental

Chemicals and reagents.—Chemicals were supplied by Sigma (St. Louis, Mo, USA). Isoamylase and Ceralpha and Betamyl substrates were obtained from Megazyme (Sydney, Australia). Expression vectors and bacterial strains were from Invitrogen (Groningen, The Netherlands). Radiotracers were supplied by Amersham–Pharmacia Biotech (Uppsala, Sweden).

Cloning and sequencing of a full-length R1 cDNA clone.—A 680 bp PCR fragment corresponding to the aminoterminal portion of R1 was used as a probe to isolate a full-length R1 cDNA clone from a potato (*Solanum tuberosum* L. cv. 'Prevalent') tuber Lambda ZAP-cDNA library (the library was a gift from Per Hofvander, Plant Science AB). The cDNA clone was sequenced by primer walking and cycle sequencing using PRISM ready reaction (Applied Biosystems) in a PCR thermal cycler. The sequencing reactions were analysed on an Applied Biosystems 373A or 377 DNA sequencer according to the instruction of the manufacturer. The sequence was assembled and edited with AUTOASSEMBLER program (Perkin–Elmer).

Growth and expression of R1 in E. coli.—A cDNA, encoding R1 without the transit peptide, was amplified with the full-length clone described above in pBluescript SK (−) as a template, and a pair of primers was designed: 5′-GTACTTACCACTGATACCTC-3′ (corresponding to the amino terminus of the processed protein) and 5′-atggccacgtcgactTCACATCTGTGGTCTTGT-3′ (corresponding to the carboxy terminal sequence TRPQM). Pfu DNA polymerase (Stragagene, USA) was used to minimise incorrect incorporation of nucleotides. Lower-case letters indicate a tail containing a *Sal*I restriction site. The PCR product was digested with *Sal*I and ligated into a *Sma*I/*Sal*I pre digested bacterial expression vector pTrxFus. The resulting plasmid pTrxHMW thus contained the R1 sequence fused to thioredoxin.

Either the pTrxFus or the pTrxHMW plasmid was transformed into *E. coli* (strain GI724, Invitrogen), and subsequently the *E. coli* strains were grown in RM-media according to manufacturer instructions (Invitrogen).

Growth and expression were performed by adding 5 mL of an overnight 28 °C culture to 600 mL induction media (Invitrogen) containing 1% glucose. After additional growth to reach $OD_{600\ nm} = 1.0$ (approximately 4 h, 28 °C, 225 rpm), L-tryptophan was added to a final concentration of 100 μg mL^{-1}. After induction, the cell culture was allowed to grow for 20 h at 28 °C, 225 rpm and the bacteria were subsequently harvested by centrifugation (2500*g*, 10 min).

SDS-PAGE.—To analyse the protein content, SDS-PAGE was carried out using 12–25% gradient gels. *E. coli* cells from 500 μL culture were harvested by centrifugation (2500*g*, 5 min), resuspended in 250 μL sample buffer, and subsequently 25 μL of this solution was applied to each lane.

Isolation of glycogen and measurements of glycogen accumulation.—Glycogen was isolated from 600 mL culture, grown as described above. The method for glycogen isolation using sonication and ultracentrifugation was essentially as described.[10]

The accumulation of glycogen during continuous growth (50 h) of the two *E. coli* strains was monitored using 50 mL aliquots taken from a 1 L culture grown as described above. Protein was measured using the method described by Bradford using BSA as standard.[25]

Determination of glycogen bound phosphate.—The amount of phosphate bound as Glc-6P in the isolated glycogen was measured after acid hydrolysis using an

enzyme coupled assay as described by Bay-Smidt et al.[26]

Analysis of glycogen structure.—To analyse the molecular structure of glycogen, isolated glycogen (500 μg) was debranched using isoamylase and chromatographed on a DX 500 system (Dionex Corp., Sunnyvale, CA) equipped with an S-3500 autosampler and fitted with a CarboPac PA-100 column as described previously.[22]

Measurements of enzyme activities.—ADPglucose pyrophosphorylase activity was measured using an enzyme coupled assay based on the method originally described by Sowokinos.[27,28] The activity of glycogen synthase was measured using 1 mM ADP-[U-^{14}C]glucose (2.2×10^{11} DPM mol^{-1}) with glycogen as primer.[29] Branching enzyme activity was measured by the iodine-staining assay with amylose as substrate.[30] One unit of enzyme activity is defined as the decrease in absorbance of 1.0 per min at 620 nm at 25 °C. The phosphorylase *a* stimulation assay was performed as described initially by Hawker et al.[29] One unit is defined as 1 μmol of glucose incorporated into α-glucans per min at 30 °C. The exo-amylase activity was measured using benzylidene blocked *p*-nitrophenylmaltoheptaoside as substrate.[31] One unit of activity is defined as the release of 1 μmol *p*-nitrophenyl per min at 25 °C.

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