Biosynthesis of bacterial glycogen and its regulation

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Bacterial glycogen biosynthesis occurs through ADP-glucose synthesis from ATP and glucose 1-phosphate and then transfer of the glucosyl moiety from ADPglucose to the pre-existing α-1,4 glucan primer. Regulation occurs at the level of ADPglucose synthesis, in contrast to mammalian glycogen synthesis, where biosynthesis occurs through UDPglucose and regulation occurs at the glycogen synthase level. Bacterial ADPglucose synthetase is a regulatory enzyme. A variation of activator specificity is observed in enzymes isolated from different bacteria. The variation has been correlated with the type of carbon assimilation or dissimilation pathways present in the organisms. Regulation of glycogen biosynthesis also occurs at the genetic level. In this review, I have attempted a survey of recent work done in the field.

GLYCOGEN has only glucose moieties bound in α-1,4 linkages and is about 10% branched via α-1,6 linkages. Many bacteria have been shown to accumulate glycogen or to contain the enzymes involved in its biosynthesis[1]. Glycogen is considered to be a storage compound that provides both carbon and energy for the microbes tha accumulate it. It is biosynthesized under limited growth conditions in the presence of excess carbon source. Glycogen is not necessary for bacterial growth, as observed with mutants carrying a deletion in a DNA segment containing structural genes for the glycogen biosynthetic enzymes[2]. In *Clostridium*, glycogen serves as an endogenous source of carbon and energy for spore formation and maturation[3]. It appears that glycogen is a reserve product and its main function in the bacterial cell is to provide both energy and carbon in periods of non-growth[4]. Here I have attempted to review critically the recent work done on the enzymatic processes involved in glycogen synthesis and its regulation at enzyme and genetic levels

Bacterial glycogen synthesis

In animals, the glucosyl donor for glycogen biosynthesis is uridine diphosphate glucose (UDPglucose). In contrast, in bacteria, the glucosyl donor is adenosine diphosphate glucose (ADPglucose), as in starch biosynthesis in plants. ADPglucose biosynthesis is catalysed by the enzyme ADPglucose synthetase (EC 2.7.7.27; ATP: α-D-glucose-1-phosphate adenylyltransferase):

$$\text{ATP} + \alpha\text{-D-glucose-1-P} \rightleftharpoons \text{ADPglucose} + \text{PPi}.$$

There is then a subsequent transfer of the glucosyl moiety from ADPglucose to a pre-existing primer that may either be an α-1,4 glucan or maltodextrin to form a new α-1,4 glucosidic linkage. This reaction is catalysed by the enzyme ADPglucose-specific glycogen synthase (EC 2.4.1.21; ADPglucose: α-1,4 glucan, α-4-glucosyltransferase):

$$\text{ADPglucose} + (\alpha\text{-1,4 glucan})_n \rightleftharpoons \text{ADP} + (\alpha\text{-1,4 glucan})_{n+1}.$$

When the α-1,4 glucan chain becomes large, a certain part of the growing chain is cleaved and transferred to another part of the glucan molecule or possibly even to another glucan molecule with the formation of an α-1,6 linkage to give a branch in the chain. This reaction is catalysed by the enzyme called branching enzyme (EC 2.4.1.18; α-1,4 glucan 6-glucosyltransferase).

Regulation of ADPglucose synthetase

In mammals, regulation occurs at the glycogen synthase level. The glycogen synthase step is a rate-limiting step in glycogenesis. Glycogen synthase is found in two forms, a dephosphorylated form called glycogen synthase I (more active form) and a phosphorylated form called glycogen synthase D (less active form, stimulated by glucose 6-phosphate). Phosphorylation occurs by protein kinases including cyclic AMP (cAMP)-dependent protein kinase[5-7]. In contrast, in bacteria, regulation of glycogen synthesis occurs at the ADPglucose synthesis level[8-10]. This is because UDP-glucose is also involved in other reactions whereas, in bacteria, the only route for ADPglucose is in glycogen biosynthesis and it is advantageous for bacteria to control synthesis of ADPglucose in order to control the synthesis of glycogen. In animals, however, UDPglucose is also involved in other reactions and it would not be advantageous to control glycogen synthesis at this level. In most systems, glycolytic intermediates are activators for ADPglucose synthesis while either AMP, ADP or P_i are inhibitors. Thus the enzyme appears to be modulated by the energy charge and requires the

presence of glycolytic intermediates for optimum activity[4]. The activator specificity of ADPglucose synthetase can vary and the enzyme may be classified into seven groups based on the observed differences in the specificity of activation by glycolytic intermediates[4,10]. This variation is possibly related to the type of carbon assimilation pathway predominant or significantly present in the micro-organism or plant cell. Preiss[4,10] has also discussed the correlation of variation of activator specificity with differences in the various carbon assimilation pathways. When the micro-organism predominantly obtains its energy via dissimilation of sugars via the glycolytic pathway, its ADPglucose synthetase is activated by fructose 1,6-bisphosphate. Both photosynthetic bacteria and plants, which assimilate their carbon via the Calvin cycle, have ADPglucose synthetases that are activated by 3-phosphoglycerate. It is suggested that the activator sites for the different groups may be related. The portion of the gene specifying the activator-binding site of the ADPglucose synthetase was modified during evolution possibly owing to pressure for coordination of the activation with the carbon assimilation or dissimilation pathway[4].

On the basis of studies with different glycogen-excess and glycogen-deficient mutants, Preiss[4,10] has also given evidence to show that the activation and inhibition phenomena observed *in vitro* are also functional *in vivo*.

Activators

In *Escherichia coli*, hexanediol 1,6-bisphosphate has been shown to be the most effective activator[11]. Fructose 1,6-bisphosphate gives the same amount of stimulation but its $A_{0.5}$ value is about 17-fold higher[12,13]. The other compounds that have been shown to activate *E. coli* ADPglucose synthetase effectively are pyridoxal 5'-phosphate and NADPH[14,15]. Various kinetic and binding studies have given evidence for binding of all the activators to the same site[16,17]. *E. coli* ADPglucose synthetase can be covalently modified with pyridoxal phosphate by reduction with sodium borohydride. After acid hydrolysis, pyridoxyllysine was isolated, indicating that the ε-amino group of lysine was involved in the binding of the allosteric activator. The modified enzyme is significantly active, with 1:1 stoichiometry of pyridoxal phosphate and enzyme[16,17].

Pyridoxylation in the absence of protectors gave a modified enzyme with only 34% of native enzyme activity, but with a low ratio of activity measured in the presence of fructose 1,6-bisphosphate to activity measured in the absence of activator (2.5 as opposed to 35 to 44 for the native enzyme)[4]. Other allosteric activators, fructose 1,6-bisphosphate and hexanediol 1,6-bisphosphate, as well as the inhibitor adenosine 5'-monophosphate (5'-AMP), protected against the inactivation caused by reductive phosphopyridoxylation. ADPglucose synthetase, even after binding pyridoxal phosphate, is still sensitive to AMP inhibition. This shows that pyridoxal phosphate and AMP bind at different sites, indicating that the allosteric-activator and inhibitor sites are distinct[4]. Protection of the enzyme from reductive phosphopyridoxylation by the allosteric effectors, along with the lowering of the ratio of activity in presence of activator to activity in absence of activator, strongly suggests that pyridoxal phosphate does bind to the activator site. Digestion of pyridoxylated enzyme and sequencing of the peptides containing pyridoxal phosphate showed that Lys-38 is responsible for binding pyridoxal phosphate. Lys-38 is in the vicinity of a segment rich in basic amino acids[16,17]. Experiments have indicated involvement of at least two basic amino acids in the binding of the allosteric activator. Chemical modification studies with phenylglyoxal have indicated the involvement of an arginine at the allosteric activator site[18].

In addition to Lys-38, it has been shown that there is another lysine important for catalytic activity. The other lysine can be protected by ADPglucose and Mg^{2+}. This lysine-containing protein segment has also been isolated and sequenced. It contains a predominant number of negatively charged amino acids and proline residues. It is considered to be the ADPglucose-binding site[19].

Since the allosteric activator-binding site is near the amino-terminal end, the N-terminal amino-acid sequences of various ADPglucose synthetases with different activator specificity have been compared[10,15,20-23]. *E. coli* and *Salmonella typhimurium* ADPglucose synthetases, which have the same activator specificity, also showed much similarity in N-terminal sequence. The N-terminal amino-acid sequences of the enzymes from the photosynthetic bacteria *Rhodospirillum tenue*, *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* are quite different from those of the *E. coli* and *S. typhimurium* enzymes and are also very much different from each other. It is interesting that the N-terminal amino-acid sequences of the enzymes from *Rhodospirillum tenue* and *R. rubrum*, which have the same allosteric-activator specificity, are quite dissimilar. Immunological tests also did not provide correlations with the activator specificity of different enzymes[23].

Rhodopseudomonas sphaeroides ADPglucose synthetase gets activated up to 5-fold by pyruvate. However, under the same conditions, bromopyruvate activates this enzyme only up to 2.2-fold. Bromopyruvate-modified ADPglucose synthetase had 2.5- to 3.0-fold higher activity than the unmodified enzyme in the absence of activator[4,24].

There is activation of ADPglucose synthetase by bromopyruvate linearly until the stoichiometry of

bromopyruvate to enzyme exceeds one. Higher concentrations, 1.3 mole bromopyruvate per mole of enzyme subunit, inhibited the enzyme activity. The addition of either activator, fructose 6-phosphate or pyruvate, to reaction mixtures with the modified enzyme caused no activation[4].

Experiments have shown that bromopyruvate binds to a cysteine residue in the enzyme forming *S*-pyruvylcysteine. Bromopyruvate gets incorporated at or near the allosteric activator-binding site. There is limited protection of the enzyme from bromopyruvate incorporation in the presence of fructose 6-phosphate and by inorganic phosphate[4].

Fructose 2,6-bisphosphate at 50 μM has no effect on either pure or partially purified ADPglucose synthetase from *Rhodopseudomonas blastica*, *Rhodospirillum molischianum*, *S. typhimurium*, *Rhodospirillum rubrum*, *Enterobacter hafniae*, *Rhodopseudomonas globiformis*, *Edwardsiella tarda*, *Shigella dysenteriae*, spinach leaf or potato tuber. However, fructose 2,6-bisphosphate has been shown to activate the *E. coli* and *Rhodopseudomonas sphaeroides* enzymes[10]. It is not as effective as fructose 1,6-bisphosphate or fructose 6-phosphate. Fructose 2,6-bisphosphate stimulates activity of the *E. coli* enzyme to the same extent as fructose 1,6-bisphosphate, but 50% activation by fructose 2,6-bisphosphate is observed at 350 μM, while only 68 μM fructose 1,6-bisphosphate is required. With the *R. sphaeroides* enzyme, the value of $A_{0.5}$ is about 9 μM for fructose 6-phosphate, 15 μM for fructose 1,6-bisphosphate and 60 μM for fructose 2,6-bisphosphate. Stimulation by fructose 2,6-bisphosphate is only about 5.5-fold, compared to 7- and 10-fold for fructose 1,6-bisphosphate and fructose 6-phosphate respectively. However, it is an open question whether bacteria synthesize fructose 2,6-bisphosphate[10].

Inhibitors

AMP, ADP and Pi have been shown to be inhibitors of many of the bacterial ADPglucose synthetases[1,25,26]. There is interaction between inhibitor and activator on the ADPglucose synthetase. Increasing concentrations of activator reverse or prevent the action of inhibitors[25-27]. This indicates that ADPglucose synthesis is controlled by excess carbon and the energy charge since ATP is the substrate of ADPglucose synthetase.

Pyrophosphate (PPi), a product of the ADPglucose synthetase reaction was earlier thought to be present in the cell in negligible concentration because of hydrolysis to inorganic phosphate. However, it has been shown that it is a normal metabolite in microorganisms[28,29]. In *E. coli*, it has been shown[29] to be present at 0.5 mM. It has also been shown that pyrophosphate acts as an inhibitor of ADPglucose synthetase[30]. ATP or the activator fructose 1,6-bisphosphate does not reverse the inhibition caused by pyrophosphate. The inhibition is additive with AMP, showing that pyrophosphate is bound in the enzyme at a site different from the AMP-binding site. Glucose 1-phosphate has been shown to reverse partially the inhibition caused by pyrophosphate, indicating mixed-type inhibition by pyrophosphate. The $I_{0.5}$ for pyrophosphate with *E. coli* ADPglucose synthetase has been determined[30] to be 40 μM. Pyrophosphate also acts as an inhibitor of ADPglucose synthetases from *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*[10].

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp) have also been shown[31] to be inhibitors of *E. coli* ADPglucose synthetase in the concentration range 0.5 to 1.5 mM. These nucleotides are considered to play a role as mediators of stringent control, a process that bacteria may use to survive when exposed to amino-acid starvation or limitation in the availability of any aminoacyl tRNA species for protein synthesis. This process also functions in depressing synthesis of RNA, phospholipids, nucleotides, polyamines and peptidoglycans, as well as in derepression of operons for synthesis of some amino acids and in depression of uptake of some metabolites[32-35]. *E. coli* strains containing a defective *relA* gene do not accumulate glycogen as effectively as *relA*$^+$ isogenic strains[36,37]. The *relA* gene is involved in the synthesis of the above guanosine nucleotides[32-35]. However, there is a significant basal level of glycogen in the *relA*$^-$ strain. Therefore it is thought that *relA* control is not absolutely required for glycogen synthesis but is rather a modulation signal in response to nutrient availability[36,37]. *relA*$^+$ Organisms accumulate glycogen and *relA*$^-$ isogenic strains do not accumulate the polysaccharide, so it was considered that pppGpp and ppGpp should behave as activators and not inhibitors. Surprisingly, *in vitro* these nucleotides have been found to act as inhibitors and not as activators[10].

Modification of *E. coli* ADPglucose synthetase with trinitrobenzene sulphonate caused loss of the enzyme's ability to be activated by fructose 1,6-bisphosphate or hexanediol 1,6-bisphosphate or to be inhibited by 5′-AMP. However, there is little loss of catalytic activity assayed in the absence of activator. Allosteric effectors protected the enzyme from trinitrobenzene sulphonate modification; substrates gave partial protection. Peptide sequencing has shown that trinitrobenzene sulphonate binds to a lysyl residue present in the N-terminal region near the activator-binding site[18].

Binding sites

Baecker *et al.*[38] have determined the complete amino-acid sequence of *E. coli* ADPglucose synthetase. Leung

and Preiss[39] have reported the complete amino-acid sequence of the *S. typhimurium* enzyme. Parsons and Preiss[17] have determined the binding site for the allosteric activators and the putative ADPglucose-binding site. Larsen *et al.*[40] used 8-azido-AMP, a photoaffinity inhibitor analogue of AMP, to determine the location of the inhibitor-binding site. They showed that Tyr-113 or Tyr-114 (if N-terminal methionine is considered as residue 1) is the major residue that binds 8-azido-AMP. This residue must therefore be involved in inhibitor binding. Lee and Preiss[41], using 8-azido-ADPglucose, showed that Tyr-113 is a major binding site for the substrate. Larsen and Preiss[42], using 8-azido-ATP, also showed the involvement of Tyr-113. Chou–Fasman analysis[43] predicted a Rossman-fold super-secondary structure[44] where the Tyr-114 residue is located[40]. On the basis of these observations, Kumar *et al.*[45] changed Tyr-114 to Phe using oligonucleotide site-directed mutagenesis, and found that the mutant enzyme had decreased enzyme activity and that this change also affected kinetic properties in relation to the substrates, inhibitor and activator. This indicates that the inhibitor-, activator- and adenine nucleotide-binding sites are proximal to each other in the enzyme's tertiary structure. Alteration of the substrate adenine nucleotide-binding site by amino-acid substitution could cause a change in the interaction of the activator-, inhibitor- and substrate-binding sites: lowered apparent affinity for substrate causes similar change in binding of activator and inhibitor. Binding studies have shown that binding of the substrate ATP alone or activator alone has no effect on binding of the inhibitor[11]. However, the presence of ATP and fructose 1,6-bisphosphate together effectively inhibits binding of the inhibitor, indicating an interplay of the three separate sites.

Mutants

A glycogen-excess mutant of *E. coli* K-12 strain 356, called *E. coli* K-12 618, was isolated by Creuzat-Sigal *et al.*[46] This mutant contains a ADPglucose synthetase affected in its regulatory properties. The enzyme is less dependent on the activator fructose 1,6-bisphosphate for activity and is less sensitive to inhibition by 5'-AMP[47]. Preiss *et al.*[14] studied the kinetic properties of another altered ADPglucose synthetase, isolated from a glycogen-excess mutant of *E. coli* B, called *E. coli* CL-1136. The enzyme is highly active in the absence of the activator and has a lower apparent affinity for the inhibitor AMP. Another glycogen-excess mutant of *E. coli* B, called *E. coli* SG-5, has an ADPglucose synthetase with higher apparent affinity for the activators fructose 1,6-bisphosphate, NADPH and pyridoxal phosphate, and a lower apparent affinity for the allosteric inhibitor AMP than the enzyme isolated from the parent strain[48].

A glycogen-deficient mutant of *E. coli* B, called *E. coli* SG-14, accumulates glycogen at only 28% of the rate observed for *E. coli* B and contains an ADPglucose synthetase with lower affinity for the activator fructose 1,6-bisphosphate[13]. Most significant is the inability of NADPH to stimulate the rate of ADPglucose synthesis. Pyridoxal phosphate is a very poor activator of the enzyme, and it was concluded that only fructose 1,6-bisphosphate functioned as an activator *in vivo*[13].

Another altered ADPglucose synthetase, with a 2-fold higher affinity for fructose 1,6-bisphosphate and a 2- to 3-fold lower affinity for the allosteric inhibitors 5'-AMP and Pi than the wild-type enzyme, was isolated and purified[49] from another glycogen-excess mutant, *E. coli* SG5-504. In the presence of the allosteric activator fructose 1,6-bisphosphate, the mutant enzyme formed oligomers with several times the tetramer molecular weight[49]. By chemical modification studies, it was shown that the mutant enzyme has one more cysteine per subunit accessible to modification by Ellman's reagent. The *E. coli* SG5-504 enzyme was inactivated more slowly than the AC70R1 enzyme by phenylglyoxal and by trinitrobenzene sulphonate. These results suggest an alteration in conformation of the SG5-504 enzyme. Carlson *et al.*[49] suggested that its ability to oligomerize in the presence of activator may be the result of a single amino-acid substitution.

Shen and Preiss[50] studied the activation and inhibition pattern of ADPglucose synthetase from *Arthrobacter viscosus*. It is activated by fructose 6-phosphate, pyruvate, deoxyribose 5-phosphate and ribose 5-phosphate. Fructose 6-phosphate was the most effective activator. The enzyme is inhibited by phosphates, sulphate, AMP, ADP, phosphoenolpyruvate, GMP and GDP. Fructose 6-phosphate completely overcame these inhibitions. Preiss *et al.*[51] studied kinetic properties of ADPglucose synthetase from *Serratia marcescens*. The enzyme showed negligible activation by fructose 1,6-bisphosphate, pyridoxal phosphate or NADPH. Phosphoenolpyruvate stimulated the rate of ADPglucose synthesis about 1.5- to 2-fold but did not affect the $S_{0.5}$ values of the substrates. AMP is a potent inhibitor of the enzyme. None of the activators could counteract the inhibition caused by AMP.

Glycogen synthase

As regards glycogen synthase, *E. coli* glycogen synthase has been purified to homogeneity and studied[52]. The bacterial glycogen synthase is similar to the mammalian enzyme. Their properties are similar in primer requirement, -SH group requirement and kinetics. However, unlike mammalian glycogen synthase, the bacterial

enzyme is not regulated either by allosteric phenomena or by covalent modification. Its sugar-nucleotide specificity is also different from that of the mammalian enzyme. The subunit molecular weight of the bacterial enzyme is about one half that of the mammalian enzyme. Binding experiments have shown that there are two sulphhydryl groups in bacterial glycogen synthase that are essential for enzyme activity. One sulphhydryl group can be modified with iodoacetic acid, and ADP or ADPglucose can protect from modification. The other sulphhydryl group can be modified with 5,5'-dithio-*bis*-(2-nitrobenzoic acid), and is protected by glycogen. Each reagent appears to be specific for a given sulphhydryl group, allowing the two groups to be distinguished[53]. We do not know about the nature of the active sites of glycogen synthase.

Branching enzyme

The *E. coli* branching enzyme has been purified to homogeneity[54]. Little is known about the amino-acid residues involved in the catalytic activity or in the binding of the polymeric substrates. The amino-acid compositions of *E. coli* glycogen synthase and branching enzyme are quite similar, although the two are not immunologically similar[55]. The N-terminal sequences of the glycogen synthase are rich in hydrophobic residues whereas the branching enzyme has a higher content of acidic and basic amino-acid residues. *E. coli* glycogen synthase and branching enzyme are antigenically very similar to glycogen synthases and branching enzymes from other enteric bacteria. However, no cross-reactivity with either enzyme in cell extracts from photosynthetic bacteria was observed.

Genetic regulation of ADPglucose synthetase

Preiss[10] and coworkers have shown that, in *E. coli* in enriched media containing 1% glucose, an 11- to 12-fold increase is observed in the specific activities of ADPglucose synthetase and glycogen synthase, and a 5-fold increase in the activity of branching enzyme. In minimal media, these enzymes are less repressed in the exponential phase of growth; still, there is 2- to 3-fold increase in the levels of ADPglucose synthetase and glycogen synthase in the stationary phase. There is no increase in branching enzyme activity in the stationary phase in mimimal media. The derepression of glycogen synthase and ADPglucose synthetase is coordinated. Cattaneo *et al.*[56] showed using chloramphenicol, that increase in the levels of glycogen-biosynthesis enzymes at the beginning of stationary phase is due to protein synthesis.

Derepression of the glycogen-biosynthesis enzymes also occurs when *S. typhimurium* LT2 (ref. 57) and *Agrobacterium tumefaciens*[58] go from logarithmic to stationary phase in enriched media. In *S. typhimurium*, the derepression of the three glycogen-biosynthesis enzymes is coordinated[57].

Preiss and coworkers[4,10,59] have isolated many glycogen-biosynthesis mutants of *E. coli* B. *E. coli* SG3 and *E. coli* AC70R1 are derepressed in ADPglucose synthetase and glycogen synthase in the exponential phase. In enriched media, mutant SG3 can accumulate twice as much glycogen per gram wet weight of cells in stationary phase and contains 8 to 11 times more ADPglucose synthetase activity and 3 to 4 times as much glycogen synthase activity than the wild-type parent strain in the exponential phase. However, branching-enzyme activity is the same. In mutant AC70R1, there is almost ten times as much glycogen accumulated in the stationary phase, and 11, 5.5 and 2.6 times as much ADPglucose synthetase, glycogen synthase and branching enzyme respectively in the exponential phase in enriched media. In both mutants, a further elevation occurs when they enter the stationary phase. In SG3, a 2- to 3-fold increase in the specific activities of ADPglucose synthetase and glycogen synthase, and in AC70R1, a 5- to 10-fold increase in the specific activities of the three enzymes are observed. The enzymes from AC70R1 and SG3 are identical with respect to kinetic constants, substrate requirement, activator specificity and inhibition properties[59,60], and immunologically also[55,59]. This indicates more normal enzyme appears to be synthesized than more active enzyme molecules.

Very little is known about the mechanism of the genetic regulation. Experiments suggested that cAMP and ppGpp may be involved in regulation of glycogen accumulation in *E. coli*. *E. coli* K-12 stringent strains that carry *relA*$^+$ can accumulate glycogen when it is starved for an amino acid. The isogenic *relA*$^-$ can accumulate glycogen poorly, suggesting that ppGpp or some *relA*-dependent metabolite is involved in the regulation of glycogen accumulation[36]. However, *relA* control is not absolute since there is a significant basal level of glycogen accumulation.

Dietzler *et al.*[61] found that addition of cAMP to cultures of *E. coli* W4597 (K) that utilize glucose as a carbon source stimulated the rate of glycogen accumulation during exponential phase of growth by up to 5-fold and in stationary phase by about 1.5-fold. cAMP had no effect on the levels of metabolites that affect ADPglucose synthetase. cAMP is also not an allosteric effector for ADPglucose synthetase. Dietzler *et al.*[61] have postulated an indirect role for cAMP in regulating glycogen synthesis by changing the cellular level of a presently unknown inhibitor of ADPglucose synthetase. Contrarily, Urbanowski *et al.*[62] and Kumar *et al.*[63] have shown that the *in vitro* expression of *glgC* and

glgA genes (structural genes for ADPglucose synthetase and glycogen synthase respectively) is stimulated 7.1- and 4-fold respectively by cAMP and cAMP receptor protein (CRP). However, expression of *glgB* gene, the gene for the branching enzyme, is not affected. They suggested a direct role for cAMP and CRP in the genetic regulation of glycogen-biosynthesis enzymes. Romeo and Preiss[64] showed that *in vitro* coupled transcription and translation of *glgC* and *glgA* was enhanced up to 26- and 10-fold respectively by cAMP and CRP. Sequence-specific binding of cAMP–CRP to a 243-base-pair restriction fragment from the region upstream from *glgC* was observed by virtue of altered electrophoretic mobility of the bound DNA[64]. The compound ppGpp significantly enhances expression of *glgC* and *glgA* by 3.6- and 1.8-fold respectively[64]. However, the ntrA and ntrC gene products, an RNA polymerase sigma factor and a DNA-binding protein respectively, showed negligible effect on the expression of these genes. These proteins activate expression of certain genes in response to nitrogen limitation. Leckie *et al.*[65] showed that in *cya* and *crp* mutants of *E. coli*, deficient in adenylate cyclase and CRP respectively, the rate of glycogen biosynthesis is lower than in the parent strain. Addition of cAMP to the media of the *crp* mutants did not give stimulation of the rate of glycogen biosynthesis.

Synthesis of the glycogen-biosynthesis enzymes is apparently under complex regulation since it is influenced not only by nutrient deprivation in the presence of excess carbon but also by the rate of bacterial growth[1,58,66]. The structural genes for the glycogen-biosynthesis enzymes are clustered at 75 min between *glpD* and *asd* genes on the linkage maps of both *E. coli*[67] and *S. typhimurium*[57]. On the basis of coordinate derepression of all three glycogen-biosynthesis enzymes during late logarithmic to early stationary phase of bacterial growth and the clustering of the *glg* genes, Preiss[4] suggested the existence of a glycogen operon in *E. coli* and *S. typhimurium*. On the basis of observations on *E. coli* mutants SG3 and AC70R1, Preiss also suggested that the expression of the *glg* genes is under the control of at least two regulatory elements in *E. coli*: SG3 has mutations in *glgR*, which is linked by P1 transduction to the structural genes involved in glycogen biosynthesis, and AC70R1 has mutations in an unlinked regulatory locus[10].

Cloning and sequencing of glycogen-biosynthesis genes

Okita *et al.*[68] isolated the structural genes by shotgun cloning of *E. coli* DNA. Large DNA fragments obtained by partial restriction-endonuclease digestion were cloned in the bacterial plasmid pBR322 and clones containing the *glg* genes were isolated by cotransformation with the neighbouring selectable gene *asd*. Earlier they used mutant G6MD3 of *E. coli* K-12, which bears a deletion in the *malA* region through *asd* and hence lacks the putative *glg* operon. One recombinant plasmid, pOP12, which contained an about 10.5-kb-long DNA fragment, has all the three *glg* genes and *asd*. Plasmid pOP12 was physically mapped with nine restriction enzymes. On the basis of deletion mapping and subcloning, the order, earlier obtained from transduction experiments, was confirmed as *asd–glgB–glgC–glgA*. There was about 2 kb between *glgB* and *glgC* and also about 1.1 kb downstream to *glgA*. Transformation of *E. coli* K-12 with pOP12 resulted in a 30-fold increase in the specific activity of ADPglucose synthetase but increased the *glgA* and *glgC* products only 8- and 10-fold respectively. Transformation of the *E. coli* B derepressed mutant AC70R1 elevated the specific activities of the three glycogen-biosynthesis enzymes about 30- to 40-fold. On the basis of electrophoresis, it was shown that the increase in activity of the glycogen-biosynthesis enzymes is due to gene-dosage effects and not due to activation of pre-existing protein[68]. Subsequently, Preiss and coworkers sequenced all the three structural genes, namely *glgA*, *glgB* and *glgC*, that encode glycogen synthase, branching enzyme and ADPglucose synthetase[38,69,70]. From the nucleotide sequences, the amino-acid sequences were deduced. The deduced amino-acid sequences are consistent with the amino-acid analysis of the purified proteins. The branching enzyme is composed of 727 amino acids, and has a molecular weight of 84,048, which is in agreement with the reported molecular weight of 84,000 (ref. 54). ADPglucose synthetase consists of 431 amino acids, and has a molecular weight of 48,762, which is also in agreement with the reported molecular weight of 50,000 (refs. 59, 60). Glycogen synthase consists of 477 amino acids, and has a molecular weight of 52,412, which is also in agreement with the reported molecular weight of 49,000 (ref. 52). Romeo *et al.*[71] also determined the complete sequence of the region between *glgB* and *glgC* and found an open reading frame capable of coding a polypeptide of 507 amino acids. However, they could not achieve expression of this open reading frame even on using the maxi-cell technique[63]. On doing a computer-assisted search for proteins similar to the putative polypeptide of *glgX*, (the open reading frame between *glgB* and *glgC*) it was found that the proposed gene product of *glgX* is similar in sequence to glucan hydrolases and transferases. The putative product is found to have significant similarity to α-amylases from mammalian and bacterial sources. Sequence alignment showed that the sequence conserved the amino acids His-122, Asp-206 and Tyr-79 of taka-amylase, which have been shown to be involved in the active site. Romeo *et al.*[71] also sequenced the

downstream region of *glgA*, which they showed to have an incomplete open reading frame with similarities with α-glucan phosphorylases. Later Yu *et al.*[72] gave the complete nucleotide and deduced amino-acid sequence of the α-glucan phosphorylase gene present downstream to *glgA*. It is interesting that the genes for glycogen biosynthesis and glycogen degradation are clustered in the *E. coli* genome.

Leung *et al.*[47] cloned the glycogen-biosynthesis genes from *E. coli* K-12 618 in plasmid pBR322. They could clone intact *glgB* and *glgC* but not *glgA*. Their recombinant plasmid, pEBL1, was physically mapped using nine restriction endonucleases. In *E. coli* K-12 618 too, the genes are clustered in the same order as in *E. coli* K-12. There is about 2 kb between *glgB* and *glgC*. Later Lee *et al.*[73] and Kumar *et al.*[74] sequenced the complete *glgC* gene of *E. coli* K-12 mutant 618 and found that, in the mutant enzyme, there are two amino-acid changes, viz. Lys-296 → Glu and Gly-336 → Asp. Using oligonucleotide site-directed mutagenesis and recombinant-DNA methods, Kumar *et al.*[74] found that the single mutation Lys-296 to Glu is without effect, whereas the mutation Gly-336 to Asp results in a defective enzyme. It is interesting that the amino acids at positions 296 and 336 are responsible for altered allosteric properties. By chemical modification studies, these amino acids in the enzyme protein were shown not to be involved in binding of substrates, inhibitor or activators. It seems that these amino acids are involved in maintaining the proper conformation of the enzyme.

Leung and Preiss[75] cloned the *glgC* and *glgA* genes from *S. typhimurium*. Subsequently, Leung and Preiss[39] also sequenced the *glgC* gene from *S. typhimurium* and deduced the amino-acid sequence. The *S. typhimurium* ADPglucose synthetase also contains 431 amino acids, and has a molecular weight of 45,580. Comparison of the *E. coli* and *S. typhimurium* LT2 ADPglucose synthetases showed 80% homology in the nucleotide sequence and 90% homology in the deduced amino-acid sequence. The amino-acid residues of putative allosteric sites for the activator fructose 1,6-bisphosphate (amino acid 39) and the inhibitor AMP (amino acid 114) are identical in the two enzymes. There is also extensive homology in the putative ADPglucose-binding site. In both *E. coli* K-12 and *S. typhimurium* LT2, the first base of the translation start codon ATG of *glgA* overlaps with the third base of the TAA stop codon of *glgC* (ref. 39).

Concluding remarks

Although work on bacterial glycogen synthesis has been carried out for more than 25 years, the genetic regulation is not clear. We do not know the exact location of the active sites of the enzymes involved. The amino-acid replacement(s) in the enzymes from various mutants of *E. coli* and *Salmonella typhimurium* are also not all known. Now, with cloned genes, oligonucleotide site-directed mutagenesis can be used to study structure–function relationships for the enzymes. X-ray crystallographic studies are also likely to reveal the amino-acid residues involved in enzyme function.

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