ELSEVIER

FEMS Microbiology Letters 194 (2001) 7–11

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Measurement of the glycogen synthetic pathway in permeabilized cells of cyanobacteria

Diego F. Gómez Casati, Miguel A. Aon, Sonia Cortassa, Alberto A. Iglesias *

Instituto Tecnológico de Chascomús (IIB-INTECH, CONICET), Camino Circunv. Laguna km 6, Casilla de Correo 164, 7130 Chascomús, Argentina

Received 11 September 2000; accepted 10 October 2000

Abstract

A simple, rapid and reliable procedure for permeabilizing cyanobacterial cells and measuring the glycogen synthetic pathway in situ, is presented. Cells from *Anabaena* sp. strain PCC 7120 were permeabilized with a mixture of toluene:ethanol (1:4 v/v). Fluorescence microscopy of cells incubated with fluorescein diacetate showed *Anabaena* non-permeabilized cells as green fluorescents, whereas permeabilized (viable) cells exhibited the intrinsic red fluorescence. Labelled α-1,4-glucan was recovered when permeabilized cells were incubated with the substrates of ADP-glucose pyrophosphorylase or glycogen synthase. The kinetic and regulatory properties of both enzymes could be reproduced in situ. The simplicity of the procedure and the ability to measure in situ glucan fluxes show the methodology as useful for studying the intracellular regulation of storage polysaccharides in a photosynthetic prokaryote.

Keywords: Glycogen; Starch synthesis; Cyanobacteria; *Anabaena* PCC 7120; Glycogen synthase; Permeabilized cell; Metabolic flux measurement; ADP-glucose pyrophosphorylase

1. Introduction

The synthesis of glycogen in bacteria and of starch in higher plants occur with the use of ADPGlc as the glucosyl donor for the elongation of an α-1,4-glucan chain [1,2]. The metabolic pathway conducting the storage polysaccharide synthesis in these organisms involves the enzymatic steps catalyzed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) and glycogen (starch) synthase (GSase; EC 2.4.1.21); followed by a branching enzyme (EC 2.4.1.18), that mediates the formation of α-1,6-linkages to render the final branched polymer. The biosynthetic route is regulated at the level of ADPGlc synthesis, being AGPase-allosterically modulated by effectors derived from the dominant carbon assimilation route in the respective organism [1,2].

AGPase from cyanobacteria was characterized as exhibiting regulatory properties similar to those found in the enzyme from higher plants [3]. In vitro, the enzyme purified from *Anabaena* PCC 7120 is mainly regulated by 3PGA (activator) and Pi (inhibitor) [3,4]. More recent studies have shown the influence of crowding agents (e.g. polyethyleneglycol) [5,6], microtubular protein [5,7] and different 3PGA/Pi ratios under crowded conditions [8], on the kinetic properties of the cyanobacterial enzyme.

Most of the characterization of the regulatory and kinetic properties of enzymes involved in storage polysaccharide metabolism in bacteria and plants was performed in non-structured, aqueous systems. Albeit that this work has led to the present knowledge on the potential role played in vivo by metabolites modulating the enzyme activity, our understanding of what actually happens in cells has been hampered by our lack of information on the real situation within the intracellular environment. Under the latter conditions, the occurrence of protein–protein (homologous and heterologous) interactions, the effective concentration of substrates and effectors, and the interaction between enzymes and macromolecules, become relevant [9]. Interactions between enzymes and cytoskeletal proteins have been shown to modify their kinetics and dynamics, and the emergence of novel enzyme behavior [9]. In fact, recent studies suggest that the kinetic and regulatory properties of cyanobacterial and plant AGPase are strongly affected by these intracellular conditions [5–8].

* Corresponding author. Tel.: +54 (2241) 430323; Fax: +54 (2241) 424048; E-mail: iglesias@criba.edu.ar

0378-1097/01/$20.00
PII: S0378-1097(00)00484-5

At least 30% of the carbon photoassimilated by plants is channelled to storage polysaccharides under optimal conditions of light, water, temperature and CO_2 concentration [10]. Thus, it becomes a timely topic to develop methods to measure the metabolic pathway and the effect of the different metabolites on the enzymes involved in glycogen/starch synthesis under intracellular environments. Cell permeabilization is a reliable method for studying in situ kinetic properties of enzymes [11,12]. Provided the fact that the enzyme is not damaged during the permeabilization procedure, information concerning kinetic parameters, sensitivity toward effectors, or the flux through a pathway can be obtained preserving the enzyme's intracellular environment [13,14]. The choice of a permeabilization procedure depends on the composition of the cell membrane and wall, as well as the stability of the enzymatic system to the permeabilizing agent [15]. Only very few studies on the permeabilization of *Anabaena* sp. have been reported in the literature [16,17].

In the present work, a simple and rapid permeabilization procedure with a mixture of toluene–ethanol is reported for autotrophically grown *Anabaena* PCC 7120 cells. The usefulness and reliability of the permeabilization procedure was tested through measurement of the glycogen synthetic pathway and the enzymes involved, i.e. AGPase and GSase. As far as we are aware, this is the first time that the glycogen synthetic pathway is measured in permeabilized cells of a photosynthetic prokaryote.

2. Materials and methods

2.1. Reagents

Radioactive material was purchased from DuPont NEN. Glc1P, ATP, 3PGA, ADPGlc, inorganic pyrophosphatase, rabbit liver glycogen and fluorescein diacetate (FDA) were from Sigma (St. Louis, MO, USA). All other reagents used were of the highest quality available.

2.2. Permeabilization of cyanobacteria

Anabaena sp. PCC 7120 cells were grown in continuous light at 30°C in BG-11 medium [18]. Cells (OD_{540} between 1.2 and 1.4) were collected by centrifuging for 2 min at $2000\times g$ and maintained for 1 h at room temperature in the dark. The cells were then resuspended in buffer 50 mM HEPES–KOH, pH 7.5, 2 mM dithiothreitol, and 0.1 mM $MgCl_2$ (buffer A). A mixture of toluene:ethanol 1:4 v/v was used to permeabilize the cells; 25 μl of the mixture was added to 500 μl of the cell suspension ($OD_{540}\sim 1.2$), and vigorously vortexed for different time periods (between 15 s and 3 min), at 25°C. The cells were centrifuged for 2 min at $2000\times g$, and the supernatant discarded. For the measurement of enzyme activity, the cells were resuspended in a small volume of buffer A, and immediately utilized for fluorescence microscopy analysis as well as for activity assays.

2.3. Fluorescence microscopy

Anabaena cells were visualized by fluorescence using a Nikon upright epifluorescence microscope, Eclipse E800, equipped with a standard filter block for fluorescein isothiocyanate, B-2A. Intrinsic fluorescence was directly visualized using intact *Anabaena* cells resuspended in buffer A. For studies of fluorescein release, intact or permeabilized cells were incubated at room temperature with 5 μM FDA in buffer A. After 30 min incubation, cells were centrifuged, washed four-fold, and resuspended in the same buffer.

2.4. Assay of glycogen synthesis from Glc1P

The consecutive enzymatic steps catalyzed by AGPase and GSase were measured in cyanobacterial permeabilized cells. The assay medium contained (unless otherwise specified): 20 μmol Mops–KOH pH 7.5, 1.25 μmol $MgCl_2$, 0.3 U inorganic pyrophosphatase, 0.5 μmol ATP and 0.1 μmol [^{14}C]Glc1P (specific activity 9.9×10^7 cpm μmol^{-1}) in a final volume of 0.2 ml. Assays were initiated by the addition of permeabilized cells (to reach a final OD_{540} of 0.6), and incubated for 4 min at 30°C. To stop the reaction and disrupt cyanobacterial cells, the incubation mixture was heated in a boiling-water bath for 2 min, vigorously vortexed and cooled on ice for about 3 min. Labelled glycogen was extracted and quantified as specified below.

2.5. Assay of glycogen synthesis from ADPGlc

The assay medium contained (unless otherwise specified): 20 μmol Mops–KOH pH 7.5, 0.02 μmol DTT, 0.4 μmol [^{14}C]ADPGlc (specific activity 7.0×10^6 cpm μmol^{-1}) in a final volume of 0.2 ml. Assays were carried out for 4 min at 30°C and initiated by the addition of the suspension of permeabilized cells (to reach a final OD_{540} of 0.6). To stop the reaction and disrupt the cyanobacterial cells, the incubation mixture was heated in a boiling-water bath for 2 min, vortexed for 1 min, and then cooled on ice for about 3 min. Labelled glycogen was then quantified.

2.6. Purification of [^{14}C]-labelled glycogen

After cooling, the samples assayed for α-1,4-glucan synthesis from labelled Glc1P or ADPGlc glycogen were purified according to [19]. A solution of 10% w/v TCA was added to each sample (final concentration 5% w/v), and then centrifuged at $10\,000\times g$ for 10 min. The TCA pellet was discarded and the supernatant mixed with two volumes of ethanol and carrier glycogen. The labelled ethanol

insoluble material was obtained by centrifugation after cooling at −20°C for 2 h. The pellet was washed twice with ethanol and measured for [^{14}C]-incorporation.

That the radioactivity was incorporated into α-1,4-glucan was corroborated through its sensitivity to amyloglucosidase (from *Aspergillus niger*). The pellet was resuspended in 0.2 ml of buffer 50 mM HEPES–KOH, pH 7.0, and incubated with an excess of the glucosidase at 30°C during 10 min. The total amount of radioactivity was recovered in the supernatant after reprecipitation of the sample with ethanol and centrifugation.

2.7. Kinetic studies

The experimental data were fitted through the generalized Hill equation by a non-linear least square regression kinetics computer program [20], and thus used to calculate kinetic parameters V_{max}, $S_{0.5}$ (concentration of substrate giving 50% of maximal velocity) and n_H, the Hill coefficient. All kinetic parameters were obtained from at least four sets of independent experiments, and are the mean of three determinations, with a reproducibility within at least ± 10%.

3. Results and discussion

3.1. Permeabilization of cyanobacterial cells

Living *Anabaena* PCC 7120 cells were visualized by fluorescence microscopy directly (Fig. 1A) or after (Fig. 1B) incubation with the fluorescent dye FDA. Cells exhibit an intrinsic (red) fluorescence, as shown in Fig. 1A. After incubation with FDA they exhibit a yellow fluorescence (Fig. 1B) due to the hydrolysis of the dye by intracellular esterase(s), rendering fluorescein [21]. Unlike FDA, the fluorescein is unable to permeate the membrane and thus intact cells retain the fluorescent compound (Fig. 1B). Fig. 1C shows that permeabilized cells incubated with FDA exhibited no fluorescein signal but intrinsic fluorescence. Indeed, effective permeabilization of cyanobacteria can be followed due to the loss it causes in the ability of cells to retain fluorescein.

The maintenance of cellular integrity is critical for the use of permeabilized cells in biological studies. We optimized the permeabilization of cyanobacteria by treating the cells several times with the solvents mixture, i.e. from 15 s up to 3 min, followed by monitoring the cellular integrity through fluorescence microscopy. After 15 s treatment, only 80% of the cells were permeabilized as could be judged by the loss of fluorescein; whereas treatments for longer than 75 s showed a disruption of cellular integrity (data not shown). Permeabilization of *Anabaena* during 1 min was the optimal time, as the integrity was conserved in cells that lost the ability to retain fluorescein after incubation with FDA (Fig. 1C).

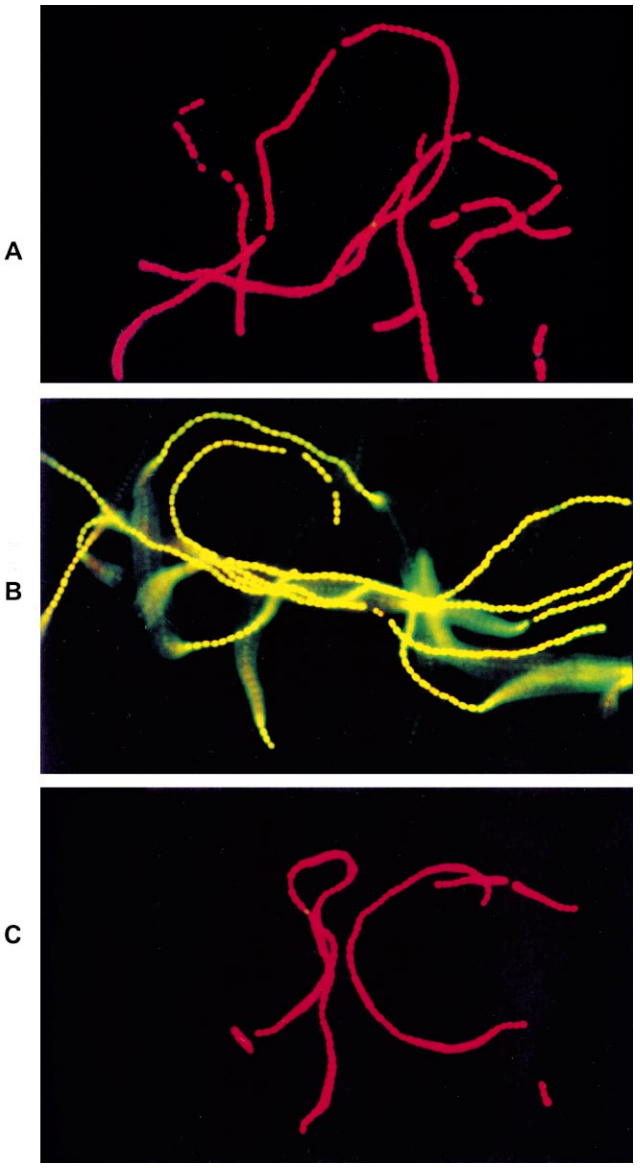

Fig. 1. Fluorescence microscopy of *Anabaena* sp. strain PCC 7120 cells. (A) Intrinsic fluorescence of *Anabaena* sp. cells. (B) Non-permeabilized cells incubated with 5 μM FDA for 30 min. (C) Permeabilized cyanobacterial cells after incubation in the presence of 5 μM FDA.

3.2. Measurement of glycogen synthesis in permeabilized cells

Another criterium to evaluate the accurate permeabilization of cyanobacterial cells was their ability to retain the biological activity of enzymes involved in glycogen synthesis. Fig. 2 shows that, in cells permeabilized during 1 min with the mixture toluene:ethanol (1:4 v/v), a radioactive label was incorporated into α-1,4-glucan from the substrates [^{14}C]Glc1P and [^{14}C]ADPGlc. In both cases, the incorporation was dependent on time of incubation with the radioactive substrate. Using permeabilized cell suspensions with an OD_{540} of 0.6 the incorporation of label from [^{14}C]Glc1P or [^{14}C]ADPGlc was linear up to 6 or 10 min,

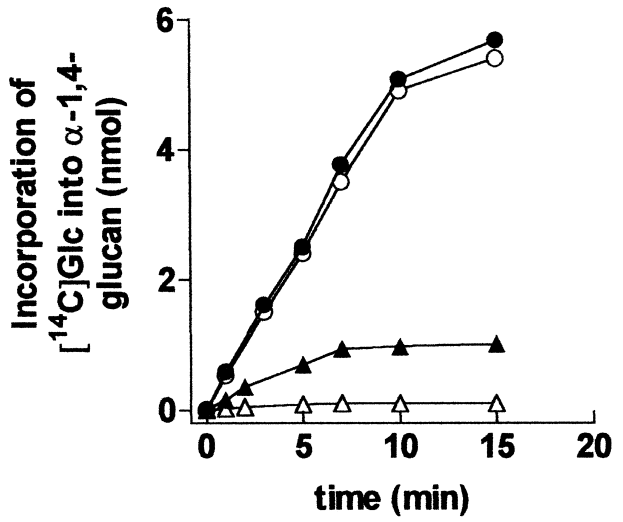

Fig. 2. Incorporation of radioactivity into α-1,4-glucan, from [^{14}C]Glc1P and [^{14}C]ADPGlc. Synthesis of glycogen from Glc1P (triangles) or from ADPGlc (circles) was measured by incubation of 0.01 ml of permeabilized cells (final $OD_{540} = 0.6$), as described in Section 2, and in the absence (empty symbols) or in the presence (filled symbols) of 2.5 mM 3PGA.

respectively (Fig. 2). Determination of initial velocities from Fig. 2 shows that label incorporation into glycogen was faster when ADPGlc was used as the substrate than when measured from Glc1P. From this, and since the measurement performed from ADPGlc corresponds to the activity of GSase, it is concluded that the incorporation of [^{14}C]Glc into the polysaccharide from [^{14}C]Glc1P is due to the activity of AGPase, which is rate-limiting in the whole process.

Incorporation of radioactive label into α-1,4-glucan from [^{14}C]ADPGlc was not affected by the presence of UDPGlc, but it was inhibited by the addition of non-radioactive ADPGlc in the assay medium (data not shown).

Table 1
Incorporation of [^{14}C]Glc into α-1,4-glucan from [^{14}C]Glc1P and [^{14}C]ADPGlc

Substrate	Activity (nmol min^{-1} ml^{-1} cells, $OD_{540} = 0.6$)			
	in situ		in vitro[a]	
	−3PGA	+3PGA	−3PGA	+3PGA
[^{14}C]Glc1P	1.7	15.3	1.4[b]	16.8[b]
[^{14}C]ADPGlc	48.6	50.2	47.1[c]	50.2[c]

Activity was assayed in permeabilized cyanobacteria or in extracts of cells disrupted by sonication in the absence or in the presence of 2.5 mM 3PGA.

[a]Assays were performed using extracts in buffer A of cells disrupted by sonication. Data are normalized to the same amount of suspended cells. Assay media were identical to those respectively utilized for in situ studies.

[b]Values of AGPase activity assayed by measuring synthesis of [^{14}C]ADPGlc as previously described [3].

[c]Values of GSase activity following the synthesis of [^{14}C]glucan by using [^{14}C]ADPGlc and glycogen from rabbit liver as substrates as described elsewhere [23].

On the other hand, synthesis of radioactive glucan from [^{14}C]Glc1P was strictly dependent on the presence of ATP (and Mg^{2+}) and it was activated by 3PGA. The latter metabolite showed no effect on the production of polysaccharide from [^{14}C]ADPGlc (Fig. 2). These results agree with reports showing that synthesis of glycogen in cyanobacteria occurs via ADPGlc being the reaction catalyzed by AGPase (and its allosteric activation by 3PGA) a key committed step in the pathway [1–4]. Thus, it is suggested that permeabilized cyanobacteria are useful as an accurate system for the study of glycogen synthesis in situ.

Further evidence on the reliability of permeabilized cells for the measure of glycogen synthesis in situ is presented in Table 1. As shown, the initial velocity of incorporation of [^{14}C]Glc into α-1,4-glucan from [^{14}C]Glc1P assayed (in the presence or the absence of 3PGA) in permeabilized cells of *Anabaena* (in situ assay) was very close to the AGPase activity assayed in extracts of identical amounts of cells disrupted by sonication (in vitro assay) (Table 1). In the same way, the measurement of α-1,4-glucan elongation in situ was correlated with the total activity of GSase found in extracts of disrupted cyanobacteria. When compared on the basis of the same amount of cells, the ratio of activities determined in situ and in vitro were close to unity in all cases (Table 1).

Table 2 illustrates the kinetic characterization of the synthesis of α-1,4-glucan by permeabilized cells from *Anabaena*. Initial velocities of the incorporation of radioactive label into the polysaccharide at varying concentrations of [^{14}C]Glc1P (in the presence of a fixed amount of 3PGA) followed a hyperbolic pattern ($n_H = 0.9$), with an $S_{0.5}$ of 0.053 mM. When [^{14}C]ADPGlc was the variable substrate, saturation curves were also hyperbolic, and the $S_{0.5}$ was calculated to be 0.45 mM (Table 2). The latter data correspond to the kinetic properties of cyanobacterial GSase determined in situ. The $S_{0.5}$ value shown in Table 2 is similar to that reported for the enzyme from different bacteria ([22]; see also the internet site http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-e+(BRENDA-ECNUMBER:'2.4.1.21')). On the other hand, the $S_{0.5}$ for Glc1P determined in permeabilized cells is very close to the value reported for AGPase purified from *Anabaena* PCC 7120 [3,8], thus showing a good relation between the kinetic parameters measured in situ and those determined in vitro.

Table 2
Kinetic parameters for the incorporation of [^{14}C]Glc from [^{14}C]Glc1P and [^{14}C]ADPGlc, in the presence of 2.5 mM 3PGA, by permeabilized cells of *Anabaena* PCC 7120

Substrate	$S_{0.5}$ (mM)	n_H	$S_{0.5}$ determined in vitro (mM)
[^{14}C]Glc1P	0.053	0.9	0.035[a]
[^{14}C]ADPGlc	0.45	1.1	0.1–0.4[b]

[a]Value previously reported for AGPase purified from *Anabaena* PCC 7120 [3,8].

[b]Values reported for GSases from different bacteria [22].

Synthetically, the present work describes a simple, rapid and reliable procedure for the permeabilization of *Anabaena* sp. PCC 7120, allowing cells maintaining their integrity in terms of cellular structure and functionality of enzymes to be obtained (at least those involved in the synthesis of α-1,4-glucan). By using the permeabilized *Anabaena* cells, the measurement of storage polysaccharide-synthesizing enzymes in situ is shown for the first time in a photosynthetic prokaryote. The system is being applied to a complete characterization of the metabolism of glycogen synthesis under conditions close to those occurring in vivo.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina, PIP 0443/98) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT 1-0674). D.F.G.C. is a fellow from CONICET; M.A.A., S.C. and A.A.I. are researchers from the same institution.

References

[1] Sivak, M.N. and Preiss, J. (1998) Starch: Basic Science to Biotechnology. Academic Press, San Diego, CA.
[2] Preiss, J. (1999) Biosynthesis of bacterial and mammalian glycogen and plant starch synthesis and their regulation. In: Biorganic Chemistry: Carbohydrates (Hecht, S.M., Ed.), pp. 489–554. Oxford University Press, Oxford.
[3] Iglesias, A.A., Kakefuda, G. and Preiss, J. (1991) Regulatory and structural properties of the cyanobacterial ADP-glucose pyrophosphorylase. Plant Physiol. 97, 1187–1195.
[4] Charng, Y.-y., Kakefuda, G., Iglesias, A.A., Buikema, W.J. and Preiss, J. (1992) Molecular cloning and expression of the gene encoding ADP-glucose pyrophosphorylase from the cyanobacterium *Anabaena* sp. strain PCC 7120. Plant Mol. Biol. 20, 37–47.
[5] Gómez Casati, D.F., Aon, M.A. and Iglesias, A.A. (1998) Molecular crowding and cytoskeletal proteins affect the allosteric regulatory properties of ADP-glucose pyrophosphorylase. In: Photosynthesis: Mechanism and Effects (Garab, G., Ed.), Vol. VI, pp. 3695–3698. Kluwer Academic Publishers, Dordrecht.
[6] Gómez Casati, D.F., Aon, M.A. and Iglesias, A.A. (1999) Ultrasensitive glycogen synthesis in Cyanobacteria. FEBS Lett. 446, 117–121.
[7] Aon, M.A., Cortassa, S., Gómez Casati, D.F. and Iglesias, A.A. (2000) Effects of stress on cellular infrastructure and metabolic organization in plant cells. Int. Rev. Cytol. 194, 239–273.
[8] Gomez Casati, D.F., Aon, M.A. and Iglesias, A.A. (2000) Kinetic and structural analysis of the ultrasensitive behavior of cyanobacterial ADP-glucose pyrophosphorylase. Biochem. J., in press.
[9] Aon, M.A. and Cortassa, S. (1997) Dynamic Biological Organization. Fundamentals as Applied to Cellular Systems. Chapman and Hall, London.
[10] Heldt, H.W., Chon, C.J., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R. and Heber, U. (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. Plant Physiol. 59, 1146–1155.
[11] Aragón, J.J. and Sols, A. (1991) Regulation of enzyme activity in the cell: effect of enzyme concentration. FASEB J. 5, 2945–2950.
[12] Cortassa, S., Cáceres, A. and Aon, M.A. (1994) Microtubular protein in its polymerized or non-polymerized states differentially modulates in vitro and intracellular fluxes catalyzed by enzymes related to carbon metabolism. J. Cell. Biochem. 55, 120–132.
[13] Clegg, J.S. and Jackson, S.A. (1988) Glycolysis in permeabilized L-929 cells. Biochem. J. 255, 335–344.
[14] Cheung, C.W., Cohen, N.S. and Raijman, L. (1989) Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. J. Biol. Chem. 264, 4038–4044.
[15] Felix, H. (1982) Permeabilized cells. Anal. Biochem. 120, 211–234.
[16] Bottomley, P.J., van Baalen, C. and Tabita, F.R. (1980) Heterocyst differentiation and tryptophan metabolism in the cyanobacterium *Anabaena* sp. CA. Arch. Biochem. Biophys. 203, 204–213.
[17] Privalle, S.L. and Burris, R.H. (1983) Permeabilization of isolated heterocysts of *Anabaena* sp. strain 7120 with detergent. J. Bacteriol. 155, 940–942.
[18] Castenholz, R.W. (1988) Culturing methods for cyanobacteria. Methods Enzymol. 167, 68–93.
[19] Aon, M.A. and Curtino, J.A. (1984) Evidence for the glycoprotein nature of retina glycogen. Eur. J. Biochem. 140, 557–566.
[20] Brooks, S.P.J. (1992) A simple computer program with statistical tests for analysis of enzyme kinetics. Biotechniques 13, 906–911.
[21] Ingham, E.R. and Klein, D.A. (1982) Relationship between fluorescein diacetate-stained hyphae and oxygen utilization, glucose utilization, and biomass of submerged fungal batch cultures. Appl. Environ. Microbiol. 44, 363–370.
[22] Yung, S.G., Paule, M., Beggs, R., Greenberg, E. and Preiss, J. (1984) Biosynthesis of bacterial glycogen: characterization of adenosine diphosphate glycose synthetases from *Enterobacter hafniae* and *Aeromonas hydrophila*. Arch. Microbiol. 138, 1–8.
[23] Eidels, L. and Preiss, J. (1970) Carbohydrate metabolism in *Rhodopseudomonas capsulata*: enzyme titers, glucose metabolism, and polyglucose polymer synthesis. Arch. Biochem. Biophys. 140, 75–89.