Does phosphorylase kinase control glycogen biosynthesis in skeletal muscle?

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Abstract Immunoblotting as well as enzyme assays demonstrate the presence of the self-glucosylating protein, glycogenin, in the protein–glycogen complex, in the sarcoplasmic reticulum and in phosphorylase kinase. In all three compartments glycogenin occurs in different, albeit, defined glucosylated forms, which upon deglucosylation are converted into a 42 kDa form. We suggest that phosphorylase kinase might have a dual function in glycogen biogenesis: firstly, control of glycogen degradation in the protein–glycogen complex via phosphorylation of glycogen phosphorylase *b*; secondly, regulation of glycogen biosynthesis on the sarcoplasmic reticular membranes via phosphorylation and thereby inhibition of glycogen synthase.

Key words: Glycogen biosynthesis; Glycogenin; Proglycogen; Skeletal muscle; Phosphorylase kinase

1. Introduction

Morphological studies first led to the concept that glycogen is synthesized near or at the SR and that glycogen granules are deposited in the interfibrillar space along the surface of the longitudinal SR [1]. Later it was shown that this granular glycogen is composed of glycogen to which glycogen-metabolizing enzymes are bound. This complex can be isolated from rabbit skeletal muscle in association with fragmented SR vesicles [2,3].

Phosphorylase kinase is the key enzyme regulating glycogen degradation. In muscle cells phosphorylase kinase is found in two different compartments: a major part of the enzyme is present in the cytosol preferentially associated with the above-mentioned glycogen particles [2,4]; a minor part is membrane bound [5,6]. Use of monoclonal antibodies has shown that all four phosphorylase kinase subunits are present at the SR [7], probably due to its high degree of farnesylation: both the α and β subunits of this hexadecameric enzyme, $\alpha_4\beta_4\gamma_4\delta_4$, carry farnesyl residues at their C-termini [8]. The dual localization of phosphorylase kinase indicates that glycogen turnover in skeletal muscle might be regulated separately in these two compartments, the SR membrane surface and the glycogen particles.

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Abbreviations: SR, sarcoplasmic reticulum; HSR, heavy SR vesicles; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel-electrophoresis.

Glycogen synthesis in rabbit skeletal muscle involves a low molecular weight form of glycogen, called proglycogen, exhibiting an apparent M_r of approximately 400 kDa [9]. Proglycogen is synthesized on a primer for glycogen synthesis which is formed on a self-glucosylating protein, glycogenin, being also present in isolated glycogen synthase in a 1:1 molar ratio [10]. The amino acid sequence of rabbit skeletal muscle glycogenin is known [11], however, limited information exists regarding the transformation from glycogenin through proglycogen to macromolecular glycogen ('classical glycogen') which has an apparent M_r of 10^7 in muscle. To get more insight into this process we looked for presence of glycogenin in the protein–glycogen complex and in the SR membranes where phosphorylase kinase is found, too.

This report demonstrates that glycogen particles isolated from rabbit skeletal muscle contain glycogenin in variable glucosylated forms; additionally, SR membranes contain an alternate form of glycogenin with an apparent M_r of 90–95 kDa which we call 95 kDa glycogenin. Surprisingly, a further glucosylated form of glycogenin with an apparent molecular mass of 200 kDa is detected in purified phosphorylase kinase.

2. Materials and methods

2.1. Preparation of HSR

HSR vesicles were isolated from rabbit back muscle as described in [12] and as modified in [13].

2.2. Preparation of protein–glycogen complex

Protein–glycogen particles were prepared as described in [2].

2.3. Preparation of phosphorylase kinase

Phosphorylase kinase from rabbit skeletal muscle was prepared as described in [14] and as modified in [15]

2.4. Protein determination

Protein was determined by [16] employing bovine serum albumin as a standard.

2.5. Western blotting

Proteins of the muscle glycogen particle or of the SR as well as phosphorylase kinase were first subjected to 10% SDS-PAGE according to [17]. The separated polypeptides were then transferred electrophoretically (160 mA for 20 h) to PVDF membrane sheets (Millipore) in a buffer containing 50 mM Tris, 50 mM boric acid, pH 8.2–8.8. Unspecific protein binding to the membrane surface was prevented by incubating and continuously shaking the sheets overnight at 4°C in TBS (200 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.3% (v/v) Tween 20 and 3% milk powder. Incubation with the primary antibody as well as with the biotin-conjugated secondary antibody was carried out at room temperature for 2 h as described in detail [7]. Following incubation at room temperature for 1 h with ExtrAvidinperoxidase (10 μl in 15 ml TBS, pH 7.4, containing 0.3% Tween 20 and 0.1% milk powder) staining was carried out in 60 ml substrate solution (30 mg

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4-chloro-1-naphtol dissolved in 10 ml methanol and a 50 ml mixture of 34.7 mM citric acid, 66.7 mM Na_2HPO_4, 20 μl 30% H_2O_2). The reaction was stopped by extensive washing with water.

2.6. Determination of enzyme activities

Activities of phosphorylase *b* [18] and phosphorylase kinase [19] were assayed on an AutoAnalyzer I (Technicon). Assays for glycogen synthase activity were carried out according to [20]: 6.8 mg HSR/ml was incubated in presence of 5 mM UDP [^{14}C]glucose (0.02 μCi/μmol), 10 mM glucose-6-phosphate, however, without addition of glycogen. During incubation at 30°C, aliquots were removed and applied to GF/C filter discs. The filter discs were washed with 10% trichloroacetic acid (twice), followed by water, acetone, then dried and counted for ^{14}C radioactivity. The self-glucosylating activity was assayed according to [21] at 6.8 mg HSR/ml in the presence of 5 μM UDP-[^{14}C]glucose and 5 mM Mn^{2+}. During incubation samples were taken and treated as described above for the glycogen synthase activity tests.

2.7. Autoradiography

Dried gels were exposed to KODAK X-OMAT XAR-5 film at −70°C for 20 days.

2.8. Materials

UDP-[$^{14}C^{(U)}$]-glucose was from NEN-DuPont. Biotin-conjugated secondary antibody (B 7014) and ExtrAvidinperoxidase (E 2886) were from Sigma. Polyclonal antibodies against glycogenin were a generous gift of Dr. Cohen, Dundee. Additionally, polyclonal antibodies raised against the glycogenin recombinant protein in goat were obtained from Dr. Whelan, Miami, and were greatly deeply appreciated. α-Amylase from human saliva (A 1031) was from Sigma. PVDF membranes were from Immobilon P., Millipore. All chemicals were of highest commercially available purity grade.

3. Results

Glycogenin has been identified by Western blotting in compartments known to be involved in glycogen turnover of rabbit skeletal muscle. In freshly isolated glycogen particles a high molecular weight component of ca. 400 kDa can be observed (Fig. 1). Upon storage this material disintegrates due to the presence of glucosidases [2]; species with apparent molecular weights of ca. 90 kDa, 80 kDa and 42 kDa are found. A final disintegration product seems to be a 42 kDa immunoreactive band which represents the 42 kDa glycogenin described before [22]. This disintegration is due to removal of glucose units from glycogenin since α-amylase treatment shifts the immunoreactive band from the approximate 400 kDa range into the 42 kDa glycogenin; additionally, several distinct intermediates of ca. 60 kDa and 50 kDa are observed (Fig. 2).

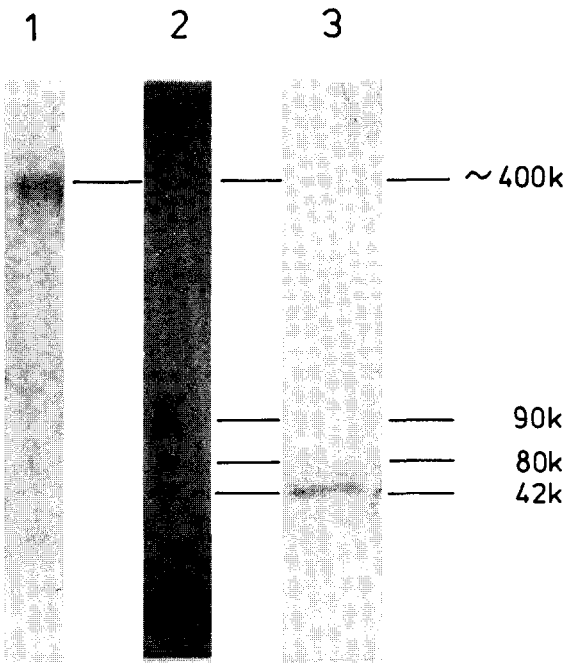

Fig. 1. Immunological demonstration of glycogenin in the protein–glycogen complex. SDS-PAGE of 70–80 μg of protein–glycogen complex and the following immunoblotting were carried out as described in section2. Lane 1, freshly prepared protein–glycogen complex; 2, after storage at −30°C for 2 months; 3, after storage at −30°C for 6 months.

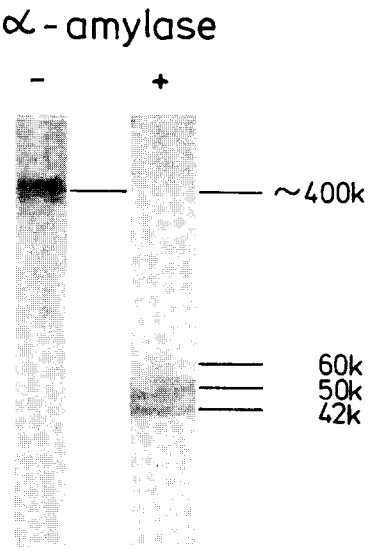

Fig. 2. Treatment of protein–glycogen complex with α-amylase. 740 μg freshly prepared protein–glycogen complex was incubated in 50 mM sodium acetate, 5 mM NaCl, pH 6.5 without (−) and with (+) 480 U α-amylase for 30 min at 20°C. SDS-PAGE of 80 μg treated sample and the following Western blotting were carried out as described in section 2.

Isolated HSR membranes show immunoreactive bands of 95 kDa and 85 kDa (Fig. 3). Again this material represents polyglucosylated glycogenin since the 42 kDa band is produced either during storage of this membrane preparation or upon incubation with α-amylase (Fig. 3). No high molecular weight material of 400 kDa could ever be detected.

Surprisingly, isolated phosphorylase kinase contains a distinct glucosylated form of glycogenin with an apparent molecular weight of 200 kDa (Fig. 4). Deglucosylation can be achieved again by incubation of the enzyme at 30°C which leads to the formation of an intermediate 95 kDa band (Fig. 4); addition of α-amylase results in the formation of a 190 kDa and later a 95 kDa band (Fig. 4). Again, the final product seems to be the 42 kDa glycogenin.

Identification of glycogenin by Western blotting in the described cellular compartments was carried out with polyclonal antibodies against the isolated glycogenin as well as with those against the recombinant protein (see section 2). In each system both antibodies identify exactly the same polypeptide(s) as an immunoreactive band(s).

The self glucosylating activity of glycogenin which transfers glucose units from UDP-glucose to the growing chain of glucose units covalently linked to glycogenin can be differentiated from glycogen synthase activity by its dependence on Mn^{2+} and

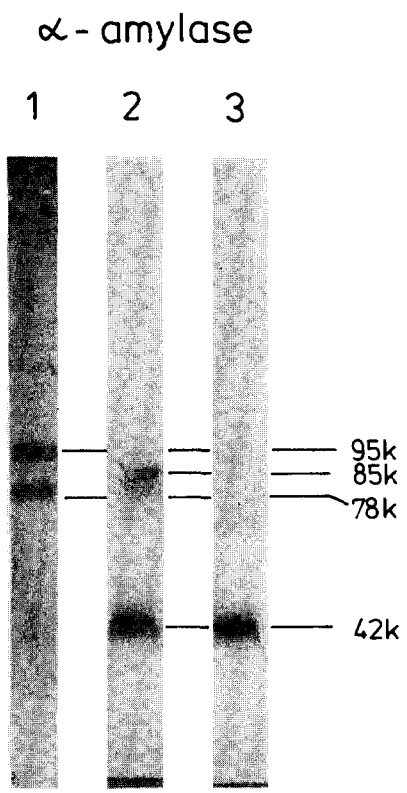

Fig. 3. Immunological demonstration of glycogenin in SR membranes. 570 μg SR membranes were incubated in absence and presence of 480 U α-amylase for 30 min at 20°C as described in the legend of Fig. 2. The following immunoblotting procedure was carried out as described in section 2. Lane 1, freshly prepared (75 μg) SR membranes; 2, 75 μg SR following incubation without α-amylase; 3, 75 μg SR following incubation with α-amylase.

its high affinity for UDP-glucose [21]. At 5 μM UDP-glucose and 5 mM Mn^{2+} essentially no self-glucosylating activity can be detected in the protein–glycogen complex nor in phosphorylase kinase (not shown). However, in HSR a rapid incorporation of glucose from the UDP-[^{14}C]glucose can be observed, followed by an immediate release of the radioactively labeled glucose (Fig. 5A). Under optimal conditions for glycogen synthase, i.e. in the presence of glucose 6-phosphate and 5 mM UDP-[^{14}C]glucose, an approximately 500-fold higher amount of glucose is incorporated which, however, is also liberated slowly (Fig. 5B). The only acceptor of [^{14}C]glucose is the 95 kDa glycogenin: autoradiography following SDS-PAGE (Fig. 6) shows that radioactively labeled glucose is detected in the 95 kDa band: inclusion of 5 mM of Mn^{2+} into the glycogen synthase assay mixture (Fig. 5B) does not change this observation, and samples taken after time points shorter than 10 min do not reveal other patterns.

In some preparations labeling in the range of 85 kDa can be detected but does not appear reproducibly and might depend on the glucosidase activity endogenously present in the SR preparations. When this glucosidase is acting the 85 kDa form of glycogenin can be formed and [^{14}C]glucose incorporation might occur. The amount of glucose incorporated by the self-glucosylating activity seems to be too low to be detectable in this autoradiographic assay.

The amount of glycogen metabolizing enzymes in HSR and for comparison in the protein–glycogen complex has been determined by assaying enzymic activities under optimal conditions and relating them to the corresponding specific activities of purified enzymes. In the protein–glycogen complex glycogen synthase makes up only ca. one-twentieth of the amount of glycogen phosphorylase whereas in SR membranes glycogen synthase is present in amounts approximately equal to that of glycogen phosphorylase (Table 1). As reported previously [7], in comparison to the amount of phosphorylase kinase in the protein–glycogen complex only a small amount of phosphorylase kinase is present in SR membranes.

Table 1
Comparison of the amount of glycogenolytic enzymes in SR membranes and in the protein–glycogen complex

Enzymes	μg enzyme · mg^{-1} protein	
	SR membranes	Protein–glycogen complex
Glycogen synthase	5	20
Phosphorylase kinase	0.1	90
Phosphorylase *b*	5	400

Activity assays were carried out as described in section 2. For the calculation of the amount of protein the following specific activities were employed: phosphorylase *b* 80 μmol · mg^{-1} · min^{-1}; phosphorylase kinase 10 μmol · mg^{-1} · min^{-1} at pH 8.2 and 10 μM free Ca^{2+}; and glycogen synthase 20 μmol · mg^{-1} · min^{-1}.

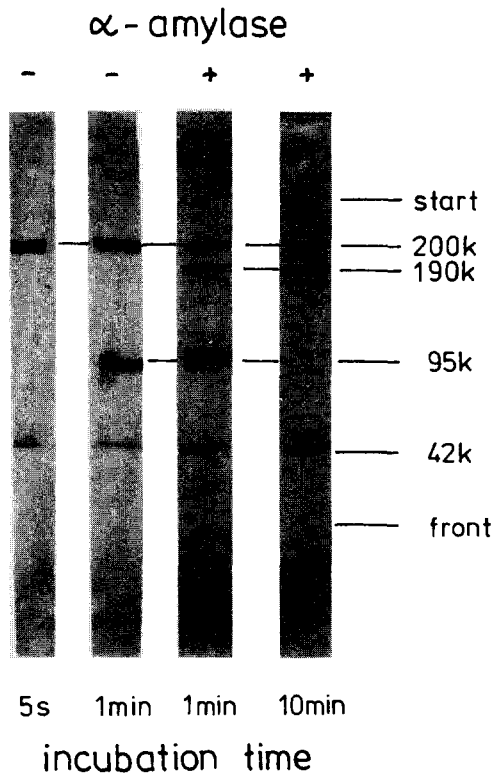

Fig. 4. Immunological demonstration of glycogenin in phosphorylase kinase. 300 μg of phosphorylase kinase, dialyzed against 50 mM of sodium acetate, 5 mM NaCl, pH 6.5, was incubated without (−) and with (+) 240 U α-amylase at 20°C. Samples were removed at time points as indicated and were subjected to SDS-PAGE. Immunoblotting was carried out as described in section 2.

4. Discussion

The idea that glycogen synthesis starts on the SR was based on morphological observations and is indeed substantiated by biochemical data presented here: glycogenin as well as glycogen synthase have been found to be associated with these membranes. This association is probably not due to a contamination of SR membranes with glycogen particles: firstly, glycogenin seems to be present in these membranes in a definitively glucosylated state represented by an apparent molecular weight of 95 kDa. In contrast, in the protein–glycogen complex glycogenin is bound in the form of proglycogen with a molecular weight of 400 kDa. Secondly, the weight ratio of glycogen synthase to glycogen phosphorylase is much higher in SR membranes than in the glycogen particles (compare Table 1). Indeed, if one counts phosphorylase *b* as a representative of the protein–glycogen complex only 0.25 μg of glycogen synthase per mg protein would be present in the SR membranes. The 25-fold higher amount indicates that glycogen synthase itself associates with SR membranes and that this enzyme is not present due to contamination of the membranes with glycogen particles. The most probable candidate responsible for this membrane association seems to be glucosylated glycogenin; it has been shown here that glycogen synthase in membranes transfers glucose units to this newly defined species of 95 kDa glycogenin.

It is well established that glycogen synthase is a substrate for phosphorylase kinase which is indeed also present in these membranes. The farnesyl residues in phosphorylase kinase do not seem to be sufficient to cause association of this enzyme with membranes. In muscle cells only a small amount of phosphorylase kinase seems to be bound to these membranes, even though all enzyme molecules are farnesylated [8]. A further signal for association of phosphorylase kinase with membranes could be the presence of the 95 kDa glycogenin. It has been shown previously that treatment of SR membranes with α-amylase prevents association of this protein kinase with membranes [23] which, as has been demonstrated here, deglucosylates the 95 kDa glycogenin to the 42 kDa form (compare Fig. 3). This form is a glycogenin molecule which contains still ca. 30 glucose residues [24]. Jennissen and Lahr [23] have already concluded that phosphorylase kinase association with membranes requires the presence of a carbohydrate moiety. Furthermore, one can postulate that phosphorylase kinase could be involved in the transfer of the 95 kDa glycogenin from the SR membranes via a 200 kDa polyglucosylated form of glycogenin into the cytosol. This specific 200 kDa form has been found to be associated with the soluble cytosolic phosphorylase kinase (compare Fig. 4). In the cytosol, further glucosylation of the 200 kDa glycogenin to proglycogen (400 kDa) could occur which would then ripen to the final 'classical' glycogen of M_r 10^7. Association of the glycolytic enzymes with this glycogen then forms the protein–glycogen complex.

A cycle will be completed if glycogen phosphorylase starts to degrade glycogen to glucose-1-phosphate which, in cooperation with a debranching enzyme, would finally liberate the 42 kDa glycogenin. Its re-association with the SR membranes could initiate glycogen biosynthesis on the SR. Consequently, phosphorylase kinase might have a dual function in this cycle: firstly, by converting phosphorylase *b* to *a*, this protein kinase regulates glycogen degradation in the glycogen particle. Secondly, phosphorylase kinase could regulate glycogen biosynthesis via phosphorylation, and thus inhibition of glycogen synthase in SR membranes. Additionally, phosphorylase kinase could be involved in the transfer of the 95 kDa glycogenin via its polyglucosylated form from the SR membranes into the cytosol.

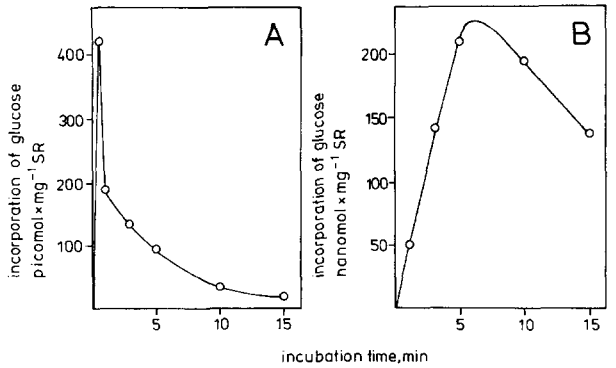

Fig. 5. Incorporation of glucose into glycogenin in SR from UDP-[^{14}C]glucose. Self-glucosylating activity and glycogen synthase activity were assayed as described in section 2. (A) Time–course of self-glucosylation of glycogenin. (B) Time–course of glucosylation of glycogenin by glycogen synthase.

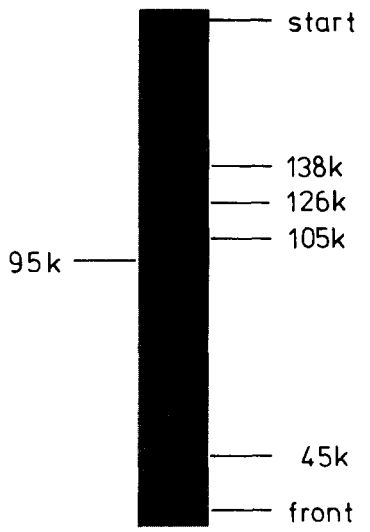

Fig. 6. [^{14}C]Glucose incorporation into 95 kDa glycogenin in SR. A sample from the assay mixture for the glycogen synthase activity after 10 min (see Fig. 5B) was subjected to SDS-PAGE according to Laemmli [17]. Autoradiography was carried out as described in section 2.

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References

[1] Wanson, J.-C. and Drochmans, P. (1968) J. Cell. Biol. 38, 130–150.
[2] Meyer, F., Heilmeyer Jr., L.M.G., Haschke, R.H. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6642–6648.
[3] Goldstein, MA., Murpha, D.L., van Winkle, W.B. and Entman, M.L. (1985) J. Muscle Res. Cell Motil. 6, 177–187.
[4] Veh, R.W., Mehnert, F.E., Petersen, J.K.H. and Jennissen, H.P. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 432.
[5] Gröschel-Stewart, U., Jennissen, H.P., Heilmeyer Jr., L.M.G. and Varsányi, M. (1978) Int. J. Peptide Protein Res. 12, 177–180.
[6] Dombradi, V.K., Silberman, s.R., Lee, E.Y.C., Caswell, A.H. and Brandt, N.R. (1984) Arch. Biochem. Biophys. 230, 615–630.
[7] Thieleczek, R., Behle, G., Messer, A., Varsányi, M. and Heilmeyer Jr., L.M.G. (1987) Eur. J. Cell Biol. 44, 333–340.
[8] Heilmeyer Jr., L.M.G., Serwe, M., Weber, C., Metzger, J., Hoffmann-Posorske, E. and Meyer, H.E. (1992) Proc. Natl. Acad. Sci., USA 89, 9554–9558.
[9] Lomako, J., Lomako, W.M., Whelan, W.J., Dombro, R.S., Neary, J.T. and Norenberg, M.D. (1993) FASEB J. 7, 1386–1393.
[10] Pitcher, J., Smythe, C., Campbell, D.G. and Cohen, P. (1987) Eur. J. Biochem. 169, 497–502.
[11] Campbell, D.G. and Cohen, P. (1989) Eur. J. Biochem. 185, 119–125.
[12] Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.-Y. and Meissner, A. (1988) Nature 331, 315–319.
[13] Hermann-Frank, A. and Varsányi, M. (1993) FEBS Lett. 332, 237–242.
[14] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[15] Jennissen, H.P. and Heilmeyer Jr., L.M.G. (1975) Biochemistry 14, 754–760.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Haschke, R.H. and Heilmeyer Jr., L.M.G. (1972) Anal. Biochem. 47, 451–456.
[19] Jennissen, H.P. and Heilmeyer Jr., L.M.G. (1974) Anal. Biochem. 57, 118–126.
[20] Nímmo, H.G., Proud, C.G. and Cohen, P. (1976) Eur. J. Biochem. 68, 31–44.
[21] Lomako, J., Lomako, W.M. and Whelan, W.J. (1988) FASEB J. 2, 3097–3103.
[22] Rodriguez, I.R. and Fliesler, S.J. (1988) Arch. Biochem. Biophys. 260, 628–637.
[23] Jennissen, H.P and Lahr, P. (1980) FEBS Lett. 121, 143–148.
[24] Lomako, J., Lomako, W.M. and Whelan, W.J. (1991) FEBS Lett. 279, 223–228.