Acylation of adenylyl cyclase catalyst is important for enzymic activity

Stefan Mollner*, Krista Beck, Thomas Pfeuffer

Department of Physiological Chemistry II, Heinrich-Heine-University, 40001 Düsseldorf, Germany

Received 3 July 1995; revised version received 24 July 1995

Abstract Incubation of human thrombocytes in the presence of [^{3}H]palmitic acid leads to incorporation of this fatty acid into the α subunit of G_s as described [Linder et al., Proc. Natl. Acad. Sci. USA 90 (1993) 3675–3679; Degtyarev et al., Biochemistry 32 (1993) 8057–8061] but also into the catalyst of adenylyl cyclase which has not been recognized before. Treatment of labeled membranes with hydroxylamine released the label from both components. Label incorporated into the catalyst could be identified as [^{3}H]palmitate. At the same time chemical deacylation caused partial loss of adenylyl cyclase activity.

Key words: Adenylyl cyclase; Palmitoylation; Chemical deacylation

1. Introduction

Fatty acylation of proteins has been recognized for more than twenty years. While cotranslational myristoylation [1,2] of proteins is an irreversible process, posttranslational attachment of palmitic acid to a cysteine residue appears to be in a dynamic equilibrium of acylation/deacylation [3–5]. Myristoylation of G-protein α-subunits at a terminal glycine was considered to provide a means for anchoring proteins in the plasma membrane [1,2] and was shown to increase the affinity of α_o for $\beta\gamma$ [6]. Another lipid modification, isoprenylation of a C-terminal cysteine residue occurs on the γ-subunit of G-proteins and was found to be necessary for interaction of $\beta\gamma$-subunits with α-subunits and effectors [7]. In general addition of long alkyl chains to proteins may allow membrane attachment thus providing high local concentration of the protein. On the other hand it may aid interaction between subunits or other protein targets.

In contrast to other α-subunits, α_s (and α_q) lacks the terminal glycine and therefore is not myristoylated, but has been recently found to incorporate palmitic acid in labile thioester bond. While the question of whether this modification regulates membrane association is still a matter of debate, there is evidence for impaired interaction with the catalyst when palmitoylation of α_s was impeded by mutational replacement of Cys-3 by Ala [8].

Besides G-protein α-subunits, G-protein linked receptors, like rhodopsin, α- and β-adrenoceptors, dopamine receptors etc. have been shown to be palmitoylated. However the consequences of palmitoylation are disparate since mutation of relevant cysteines sometimes leads to loss of stimulation of effector and prevention of desensitisation (β_2-receptor [9]) but in other cases fails to perturb receptor-G-protein coupling (α_{2A}-receptor [10]). Finally, depalmitoylation of membrane bound rhodopsin can even improve activation of its G-protein transducin [11].

The present study is aimed at a possible acylation of the effector part of the adenylyl cyclase system. We wanted to study deacylation by hydroxylaminolysis, because this occurs without damage of the protein and further more may be of advantage over the mutational approach eventually causing conformational changes.

2. Materials and methods

2.1 Materials

Guanosine 5′-(3-thiotriphosphate), (GTPγS), creatine phosphate, creatine kinase, alkaline phosphatase, activated, were obtained from Boehringer (Mannheim); Pansorbin (*Staphylococcus aureus* cells) was from Calbiochem; Trasylol was from Bayer; protein A and CSPD were from Serva; forskolin was a generous gift from Dr. H. Metzger, Hoechst (Frankfurt). All other chemicals were of the highest purity commercially available. [α-^{32}P]ATP (760 Ci/mmol) and [^{3}H]cAMP (15-30 Ci/mmol) were obtained from Amersham Buchler (Braunschweig); [^{3}H]palmitic acid (60 Ci/mmol) was from Biotrend (Köln).

2.2 Buffers

Buffer A: 10 mM MOPS, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 0.02% NaN_3, 10 μM benzethonium chloride, 200 μM PMSF, 2.8 μg/ml Trasylol, pH 7.0; Buffer B: 10 mM MOPS, 1% sucrose, 1 mM EDTA, 1 mM EGTA, 50 μM benzethonium chloride, 2.8 μg/ml Trasylol, 40 μM PMSF, 0.1 mM DTE, 1 mM benzamidine pH 7.4; Buffer C. 10 mM MOPS, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM Tween 60, 100 mM NaCl, Buffer D: 10 mM HEPES, pH 7.4, 140 mM NaCl, 2 5 mM KCl, 0 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 5 mM glucose.

2 3. Preparation of platelet membranes

Fresh platelets from healthy donors were obtained from DRK (Breitscheid) and membranes prepared therefrom as described in [13] and stored at −70°C.

2 4 Immunochemicals

Antibody BBC-4 against the adenylyl cyclase catalyst was prepared and coupled to Pansorbin cells as described [14]. For immunodetection, antibody BBC-4 was coupled to alkaline phosphatase by a procedure suggested by the distributor. CSPD was used as substrate for the alkaline phosphatase, the product of which was detected by chemiluminescence. Antibody GSA 379 against α_s was raised in rabbits according to Palm et al. [15].

2.5. Assays

Adenylyl cyclase activity was measured in 30 mM MOPS, pH 7.4, 10 mM creatine phosphate, 100 μM GTP, 20 μg/ml creatine kinase, 4.5 mM theophylline, 2 mM $MgCl_2$, 1 mM dithiothreithol, 2 mg/ml bovine serum albumin, 0.1 mM [α-^{32}P]ATP ($1–2 \times 10^6$ cpm/test tube) for 20 min at 30°C. Protein estimation was performed according to Lowry et al. [16].

3. Results

Fig. 1. shows different functions of human platelet adenylyl

*Corresponding author. Fax: (49) (211) 311 2726.

Abbreviations G_s, stimulatory guanine nucleotide binding protein; α_s, α-subunit of G_s; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate).

SSDI 0014-5793(95)00864-0

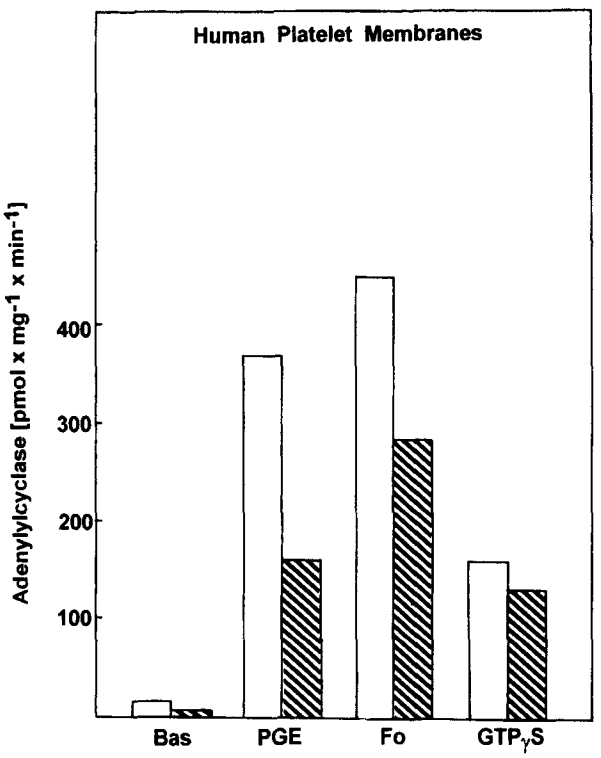

Fig. 1. Effect of hydroxylamine on human platelet adenylyl cyclase. Membranes (10 mg/ml) from platelets in buffer A were incubated with 1 M NH_2OH/HCl (hatched bars) or 1 M NaCl (open bars), adjusted to pH 7.0, for 30 min at 25°C. Following washing twice with 20 volumes of buffer B and centrifugation at 30,000 × g for 10 min at 4°C, platelet membranes (70 μg/test) were assayed for adenylyl cyclase activity in the absence (Bas) and presence of 100 μM forskolin (Fo) or 10 μM PGE_1 or 100 μM GTPγS plus 10 mM $MgCl_2$ (GTPγS). The data are representative for at least seven independent experiments.

cyclase after incubation of membranes with 1 M neutral hydroxylamine at 25°C. As is evident, basal, PGE- and forskolin-stimulated activities were decreased by 70, 60 and 40%, respectively. Surprisingly, stimulation by stable GTP-analogs was less affected than other functions. The reason for this is not entirely clear. However recent studies in this laboratory on chemical deacylation of α_s in various membranes point to an enhancement of reconstitutive activity (Mollner, S., unpublished).

Qualitatively similar results on adenylyl cyclase activity were obtained when rabbit myocardial membranes were treated with hydroxylamine (not shown).

While the loss of forskolin-stimulated adenylyl cyclase activity on hydroxylamine treatment has already suggested impairment of the catalytic function (Fig. 1), experiments in Fig. 2 were supporting this. Here, activated pure G_s (Fig. 2A) was reconstituted with solubilized catalyst from thrombocytes. Again a significant decline in activity was apparent if membranes had been subjected to hydroxylaminolysis prior to solubilisation. For comparison stimulation by forskolin (Fig. 2B) is shown which was reduced to the same degree as in membranes. The effect on the soluble catalyst was not due to differential solubilisation as shown by Western blot analysis. The loss in catalyst activity on hydroxylaminolysis was also observed with myocardial and erythrocyte membranes (not shown).

Finally we were interested to learn whether the effects on catalytic activity could be correlated with loss of protein bound acylate, preferably palmitate because of the observed sensitivity to hydroxylamine treatment. As a consequence we looked at incorporation of palmitic acid into the catalyst, as a hitherto unknown target of fatty acylation. For comparison we have monitored palmitoylation of α_s, already demonstrated in a variety of other tissues. We treated fresh human thrombocytes with 5 μM [^{3}H]palmitic acid, solubilized plasma membranes with SDS-containing RIPA-buffer and immunoprecipitated the catalyst and as a reference, the α_s moiety with the respective specific antibodies as described previously [14,17,18]. Analysis by SDS-PAGE confirms [^{3}H]palmitate-incorporation into α_s (Fig. 3A) but reveals [^{3}H]acylation of the catalytic protein as well (Fig. 3B). The dotted lines indicate complete loss of label after treatment of membranes with hydroxylamine, suggesting that the fatty acid residue had been bound within a labile thioester bond. The upper pannels represent Western blots of gels run in parallel indicating that hydroxylaminolysis did not cause loss of antigen in either case. Regarding the individual recoveries of both components after immunoprecipitation, the absolute amount of incorporated ^{3}H into α_s was about three times that into the catalyst. This roughly matches the estimated abundance of these components in platelet membranes, obtained by quantitative Western blot analysis (α_s) and approximation of amount of catalyst from enzymatic activity [18] and the turnover number of the pure catalyst [20].Fig. 4 compares removal of ^{3}H from the 150 kDa catalyst and loss of forskolin-stimulated activity as a function of hydroxylamine concentration. The results indicate that both events may be interdependent. Identification of the type of fatty acid incorporated into the catalytic protein revealed that almost 100% of the ^{3}H label was released by NaOH and comigrated exclusively with palmitic acid when analysed by HPLC on an RP-18 column (Fig. 5).

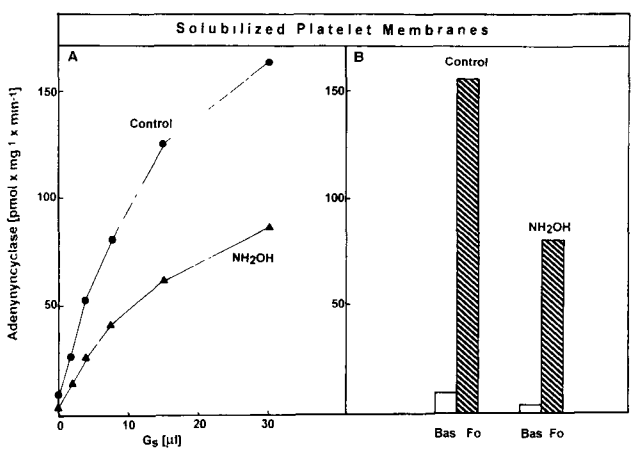

Fig. 2. Influence of hydroxylamine-treatment on reconstitution of the catalyst with α_s. Membranes from platelets in buffer A (10 mg/ml) were treated with 1 M NH_2OH/HCl or 1 M NaCl for 30 min at 25°C, washed with buffer B (2 times with 20 volumes). Adenylyl cyclase from treated and control membranes was solubilized with cholate as described in [13] with the exception that 0.6% instead of 3% cholate was used. (A) Aliquots (10 μl) of solubilized membranes in buffer C were reconstituted in the adenylyl cyclase assay with variable amounts of pure G_s (turkey erythrocytes, 1 ng/μl) or were assayed in the presence of 100 μM forskolin (B). (Bas = basal activity, Fo = forskolin). The data are representative for three experiments.

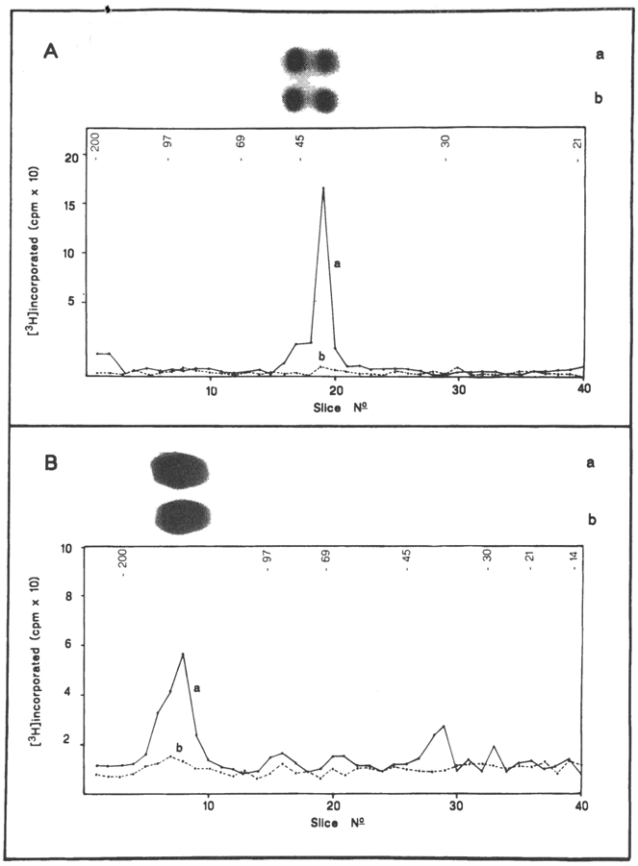

Fig. 3. Incorporation of [^{3}H]palmitate into α_s (A) and into the catalytic unit (B) of adenylyl cyclase. Human platelets, resuspended to a final concentration of 10^9 cells/ml in buffer D containing 3.6 mg/ml fatty acid free bovine serum albumin, 0.3 μM iloprost and 1 U/ml apyrase were incubated in the presence of 5 μM [^{3}H]palmitate (300 μCi/ml) at 37°C for 120 min. Platelets were washed twice with 6 ml each of buffer D and lysed as described in [13]. Membranes (10 mg/ml) were resuspended in 10 ml of buffer A plus 1 M NH_2OH/HCl or 1 M NaCl, left for 30 min at 25°C, and washed with 0.5 ml of buffer B (3 times) by centrifugation at 30,000 × g for 15 min at 4°C (A) For immunoprecipitation of α_s, labeled membranes (1.2 mg) were solubilized with 500 μl of RIPA buffer as described [14,18], followed by addition of 100 μl affinity-purified anti-α_s-antibody (against C-terminal amino acids 379–397) After incubation for 150 min at 4°C, 70 μl of 30% Pansorbin in RIPA buffer were added and left for 75 min at 4°C The washed immunoprecipitate [14,18] was treated with 100 μl Laemmli-buffer [17] at 80°C, centrifuged and applied to an 11% SDS-polyacrylamide-gel. For estimation of incorporated [^{3}H]palmitate, dried gels were cut into 2 mm slices and incubated with 150 μl water and 750 μl tissue solubilizer TS-1 (Zinsser) for 180 min at 50°C. After cooling, 12 ml of scintillant were added for counting. Tracks a and b represent ^{3}H incorporation without (——) or with subsequent (---) hydroxylamine treatment. The insert shows a Western blot run in parallel for detection of α_s [18]. The α_s migrates with an M_r of 42 kDa; the 50 kDa band corresponds to the heavy chain of IgG which is also detected by [^{125}I]Protein A. (B) For immunoprecipitation of the catalytic subunit, labeled membranes (1 mg) were solubilized in 500 μl RIPA buffer, mixed with 100 μl 30% BBC-4-Pansorbin conjugate and agitated for 180 min at 22°C. Following washing [10,16] of the immunoprecipitate, bound protein was released at 50°C with 100 μl of Laemmli-buffer [19] and subjected to polyacrylamide gelelectrophoresis (5–15%) as described [14,18] For estimation of incorporated [^{3}H]palmitate, gels were processed as in A. Tracks a and b represent ^{3}H incorporation followed by treatment with hydroxylamine (---) or not (——). The insert shows Westernblots run in parallel for detection of catalyst [14,18] using an antibody BBC-4/alkaline phosphatase conjugate and CSPD as chemiluminescence substrate The data are representative for two experiments with each separately labeled cells.

4. Discussion

This paper presents evidence that the hydroxylamine-induced loss of forskolin stimulated and reconstitutive adenylyl cyclase activity on recombination with α_s may be explained by removal of one or more fatty acyl residues from the catalyst itself. Reversible palmitoylation of the latter could indeed be demonstrated following incubation of intact cells with [^{3}H]palmitic acid. Fig. 3 shows that the ^{3}H label which is precipitated by a monoclonal antibody recognizing a common motif of eukaryotic adenylyl cyclases (Mollner, S., unpublished) migrates as a broad 150 kDa band on SDS gels, shape and M_r being consistent with those of adenylyl cyclase from human thrombocytes [18]. This is to our knowledge the first demonstration of a fatty-acid modified effector in a G-protein mediated signal tansduction chain. In this respect it is also of interest that adenylyl cyclase from *Bordetella pertussis* was found to be amidically palmitoylated, a modification obviously necessary for effective entry into the host cell [21].

The role of acylation of eukaryotic adenylyl cyclases remains to be clarified. Forthcoming studies have also to show which subtypes of the enzyme are bearing this modification. The fact that adenylyl cyclases from other tissues, normally harboring several isoforms, are likewise deactivated by hydroxylamine treatment, points to a more common property. Of course a macromolecule like adenylyl cyclase catalyst spanning 12 times the bilayer does not need additional attachment to the membrane. However, one or the other of the two large cytosolic domains, both comprising M_rs of 40 kDa, may form an extra loop necessary to maintain a tertiary structure favourable for interaction with regulatory proteins and/or for optimal catalytic efficiency. Further investigations including chemical and

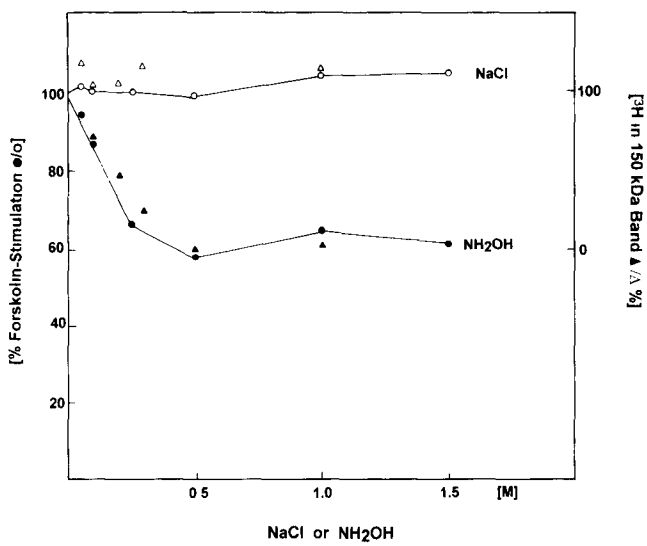

Fig. 4. Correlation between NH_2OH induced losses of catalyst-bound [^{3}H]palmitate and cyclase activity. Platelets were labeled with [^{3}H]palmitate as described in the legend to Fig. 3. Membranes were prepared and 1 mg (in 100 μl) each, was treated with various concentrations of NH_2OH/HCl (●, ▲) or NaCl (○, △) as described in legend for Fig. 3. 800 μg of protein each were used for measuring [^{3}H]palmitate bound to the catalyst (▲, △) in gelslices 1–12 (see Fig. 3). A background level of 10–15 cpm/slice was subtracted. Adenylyl cyclase activity (100 μM forskolin) was estimated in parallel using 40 μg of membrane protein (●, ○).

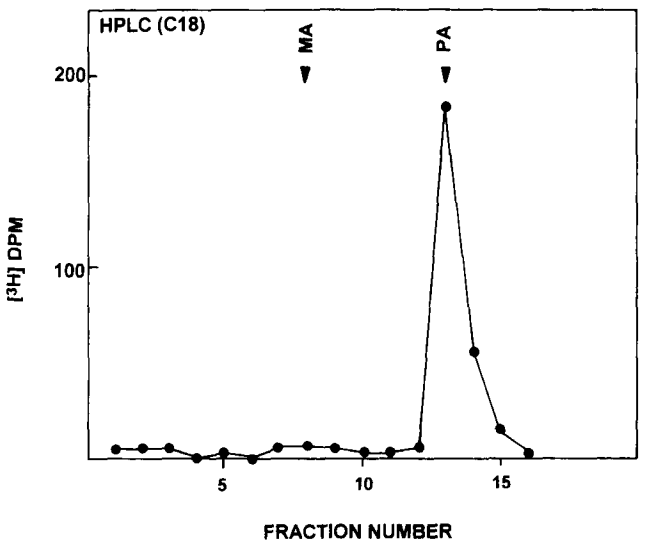

Fig. 5. Identification of [^{3}H]fatty acid bound to the catalyst. Incorporation of [^{3}H]fatty acid was performed as elaborated in legend to Fig. 3. Following blotting onto the PVDF membrane, the relevant region was excised and treated with 0.75 ml 1 M NaOH for 30 min at 25°C. Subsequent to addition of the same volume of 1 M HCl and 75 μl of 2 M glycine HCl buffer pH 3.2, the mixture was extracted with 1.5 ml n-hexane. The organic solvent was then concentrated and chromatographed on an RP C-18 HPLC column (Merck) developed with 80% (v/v) acetonitrile plus 17 mM orthophosphoric acid (MA = myristic acid, PA = palmitic acid).

molecular biological methods to localize the acylated cysteine(s) within the amino acid sequence of adenylyl cyclase are on the way.

References

[1] Mumby, S.M., Heukeroth, R.O., Gordon, J.I. and Gilman, A G. (1990) Proc. Natl. Acad. Sci. USA 87, 728–732.
[2] Jones, T.L.Z., Simonds, W.F., Merendino Jr., J.J., Brann, M.R. and Spiegel, A.M. (1990) Proc. Natl. Acad. Sci. USA 87, 568–572.
[3] Degtyarev, M.Y., Spiegel, A.M. and Jones, T.L.Z. (1993) J Biol. Chem. 268, 23769–23772
[4] Wedegaertner, P.B. and Bourne, H.R. (1994) Cell 77, 1063–1070
[5] Mumby, S M., Kleuss, Ch. and Gilman, A.G. (1994) Proc. Natl. Acad. Sci. USA 91, 2800–2804.
[6] Linder, M.E., Pang, I.H., Duronio, R.J., Gordon, J.I., Sternweis, P.C. and Gilman, A.G. (1991) J. Biol. Chem. 266, 4654–4659.
[7] Iniguez-Lluhi, J A., Simon, M.I., Robishaw, J.D. and Gilman, A.G (1992) J. Biol. Chem. 26, 23409–23417.
[8] Wedegaertner, P.B., Chu, D.H., Wilson, P.T., Levis, M.J. and Bourne, H.R. (1993) J Biol. Chem. 268, 25001–25008.
[9] O'Dowd, B.F., Hnatowich, M., Caron, M G., Lefkowitz, R.J. and Bouvier, M. (1989) J. Biol. Chem. 264, 7564–7569.
[10] Kennedy, M.E. and Limbird, L.E. (1993) J. Biol. Chem. 268, 8003–8011.
[11] Weiss, E.R., Osawa, S., Ski, W. and Dickerson, C.D. (1994) Biochemistry 33, 7587–7593.
[12] Morrison, D.F., O'Brien, P.J. and Peperberg, D.R. (1991) J. Biol Chem. 266, 20118–20123.
[13] Mollner, S., Deppisch, H. and Pfeuffer, T. (1992) Eur J. Biochem. 210, 539–544.
[14] Mollner, S., Simmoteit, R., Palm, D. and Pfeuffer, T. (1991) Eur. J. Biochem. 195, 281–286.
[15] Palm, D., Münch, G., Malek, D , Dees, C. and Hekman, M. (1990) FEBS Lett. 261, 294–298.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) J. Biol. Chem. 193, 265–275.
[17] Mollner, S. and Pfeuffer, T. (1988) Eur. J. Biochem. 171, 265–271.
[18] Simmoteit, R., Schulzki, H.D., Palm, D., Mollner, S. and Pfeuffer, T. (1991) FEBS Lett. 285, 99–103.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Pfeuffer, E., Dreher, R.M., Metzger. H. and Pfeuffer, T (1985) Proc. Natl Acad. Sci USA 82, 3086-3090.
[21] Hackett, M., Guo, L , Shabanowitz, J., Hunt, D.F. and Hewlett, E L. (1994) Science 266, 433–435.