Reversible ADP-ribosylation of the 78 kDa glucose-regulated protein

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Starvation of Mouse hepatoma cells for essential amino acids or glucose results in the mono-ADP-ribosylation of the 78 kDa glucose-regulated protein, GRP78. Here we show that the ADP-ribosylated and non-ADP-ribosylated forms of GRP78 are interconvertible during tryptophan starvation and refeeding. In addition, the ADP-ribosylation of GRP78 was shown to be reversible even during nutritional stress. The overexpressed pool of non-ADP-ribosylated GRP78 synthesized during tunicamycin treatment was available for ADP-ribosylation during subsequent amino acid starvation, especially in the absence of tunicamycin. The reversible ADP-ribosylation of GRP78 could be part of a metabolic control mechanism in operation during nutritional stress.

ADP-ribosylation; GRP78/BiP; Tunicamycin; Amino acid starvation; Nutritional stress

1. INTRODUCTION

Mono-ADP-ribosylation is a posttranslational modification of proteins employed by a variety of microbial toxins to modify the metabolism of target cells. Toxin transferases use NAD as a substrate for the addition of ADP-ribose to certain GTP-binding proteins resulting in altered enzymatic activity of the modified proteins [1–3]. Such a mechanism has the potential of being of importance in the physiological regulation of cellular metabolism, particularly if the process is reversible. Precedence for a role of ADP-ribosylation in metabolic regulation has come from studies of nitrogen fixation in bacteria [4,5]. In the reduction of molecular nitrogen to ammonia, the enzyme dinitrogenase becomes oxidized. It is regenerated through reduction by dinitrogenase reductase. The activity of this latter enzyme is inhibited in the presence of fixed nitrogen such as ammonia [5] or glutamine [6]. The mechanism of inhibition in *Rhodospirillum rubrum* is by mono-ADP-ribosylation of a specific arginine residue of dinitrogenase reductase [7].

The 78 kDa glucose-regulated protein, GRP78, is a member of the 70 kDa heat shock family of stress proteins and is believed to participate in the assembly of secretory and integral membrane proteins [8–12]. GRP78 is overexpressed in cells which are producing aberrant proteins as a result of interference with post-translational processing of secretory proteins [13]. GRP78 has been shown to be modified by ADP-

riosylation [14,15]. The ADP-ribosylation occurs during nutritional stress, either amino acid starvation or glucose starvation [16]. The data presented here demonstrate that the ADP-ribosylation of GRP78 is reversible even during nutritional stress and suggest that this may be a component of a regulatory mechanism which adjusts a cell's metabolism in accordance with the availability of nutrients.

2. MATERIALS AND METHODS

2.1. Cell culture and harvesting

The mouse hepatoma cell line, Hepa, was grown in Dulbecco's-modified Eagle's medium (DME, K.C. Biological, Kansas City) supplemented with 2% fetal bovine serum (K.C. Biological) and maintained in a humidified atmosphere of 5% CO_2/95% air. Experimental media were made from salts (Fisher), vitamins and amino acids (Sigma) according to the recipe supplied by K.C. Biological. All experimental media were supplemented with 2% dialyzed fetal bovine serum (K.C. Biological). Streptomycin (100 μg/ml, Sigma) and penicillin (100 U/ml, Sigma) were added to all media.

Hepa cells were grown to approximately 75% confluency in 60-mm culture dishes. Cultures were incubated in fresh complete medium (DME) for 2 h. Medium was removed and the cultures were washed with Puck's saline A (NaCl, 200 mM; KCl, 5 mM; $NaHCO_3$, 4 mM; glucose, 5.5 mM). Complete, tunicamycin-treated or amino acid-deficient media were added as appropriate for each experiment. Media were removed and the cultures were washed with Puck's saline A. Cells were scraped from the dish in 300 μl 2% NP-40 (Nonidet P-40, Sigma) and homogenized with 10 strokes in a glass/glass homogenizer at 4°C. Homogenates were centrifuged at 10 000 × g for 10 min. Postmitochondrial supernatants were centrifuged at 50 000 rpm for 60 min in a Ti50 rotor (Beckman) to prepare postribosomal supernatants. The supernatants were collected and stored at −80°C.

2.2. One- and two-dimensional polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done on 7.5% acrylamide gels run in the Laemmli buffer system [17]. Two-dimensional (2-D) SDS-PAGE was performed ac-

cording to O'Farrell [18]. Isoelectric focussing was done in 1 ml disposable glass pipettes using Resolve ampholytes (Isolabs) pH 3–7. The second dimension was as described for SDS-PAGE. Gels were fixed and stained with 0.1% Coomassie brilliant blue R250 (Sigma) in 50% methanol/7% acetic acid (Fisher Scientific). Gels were destained in 5% methanol/7% acetic acid.

2.3. Liquid scintillation counting of 2-D gel plugs

Radiolabeled proteins purified by 2-D SDS-PAGE were dissolved according to the method of Donato et al. [19]. Following fixing and staining of the 2-D gels, gel spots were excised and placed in scintillation vials containing 50 μl 30% H_2O_2 (Sigma) and 5 μl of 10 mM $CuSO_4$ (Baker). The vials were tightly capped and incubated for 16 h at 37°C. 200 μl of deionized water and 3 ml of Ready-Solv EP scintillation cocktail (Beckman) were added and the samples counted with a Minaxi Tri-Carb 4000 series scintillation counter (Packard).

3. RESULTS

3.1. Interconversion of the ADP-ribosylated and non-ADP-ribosylated forms of GRP78

It was previously shown that refeeding of cultures labeled with [³H]adenosine resulted in the disappearance of label from GRP78 [15]. Either ADP-ribose was being removed from GRP78 or the ADP-ribosylated GRP78 was being degraded. In order to distinguish between these two possibilities, cultures of Hepa cells were pulse-labeled with [³⁵S]methionine, chased in tryptophan-free (starvation) medium containing excess methionine, and harvested at various times. The remaining starved cultures were refed control medium with excess methionine and harvested at hourly intervals. Samples were analyzed by 2-D SDS-PAGE. We have previously shown that ADP ribosylated and non-ADP-ribosylated forms of GRP78 can be distinguished from one another on the basis of differing electrophoretic mobilities on 2-D SDS-PAGE gels stained with Coomassie blue [16] (Fig. 3). The distribution of label between the ADP-ribosylated and the non-ADP-ribosylated forms of GRP78 was measured during starvation and refeeding by scintillation counting of excised and dissolved 2-D gel plugs. Fig. 1 shows the rapid starvation-dependent accumulation of label in the ADP-ribosylated form of GRP78 with a concomitant decrease of label from the non-ADP-ribosylated form. Upon refeeding label disappears from GRP78 and reappears in the non-ADP-ribosylated form. The rate of disappearance obeys 1st order kinetics and has a half-life of approximately 3 h. These data support the notion that the ADP-ribosylation of GRP78 is a reversible process. Label decays from the total GRP78 pool with an apparent half-life of 20 h. A similar rate of decay is observed under fed conditions (data not shown) indicating that the rate of turnover of total GRP78 is unaffected by starvation.

3.2. ADP-ribosylation of GRP78 during amino acid starvation

In previous studies it was observed that the extent of the ADP-ribosylation of GRP78 never exceeded 50%
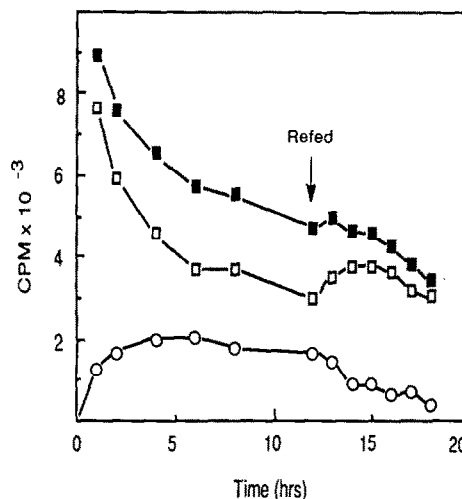

Fig. 1. The metabolic interconversion of GRP78 and ADP-ribosylated GRP78 during starvation and following refeeding. Following a 2 h incubation in complete medium, cultures were washed with Puck's saline A, and methionine-free medium containing 50 μCi/ml [³⁵S]methionine (NEN, 1134 Ci/mmol) was added for a 30 min pulse. Cultures were washed with Puck's saline A and rinsed with complete (control) medium containing a 10-fold excess of cold methionine. Twelve cultures were chased with tryptophan-free medium (starvation) containing excess methionine. Six starved cultures were harvested following 1, 2, 4, 6, 8 and 12 h chase. Six of the starved cultures were refed control medium containing excess methionine following 12 h of starvation (Refed). These cultures were harvested at 13–18 h chase and represent 1–6 h of refeeding. Postmitochondrial supernatants were stored at −80°C. Samples were analyzed by 2-D SDS-PAGE and the spots corresponding to GRP78 and ADP-ribosylated GRP78 were excised, dissolved and counted. Total GRP78, (■—■); non-ADP-ribosylated GRP78, (□—□); ADP-ribosylated GRP78, (○—○).

[16]. Either there were two distinct pools of GRP78, one available for ADP-ribosylation and the other not, or the reaction was reversible and the observed distribution represented an equilibrium situation. Hepa cells were starved for tryptophan for up to 12 h. During the last two hours of the starvation period, [³H]adenosine was added to the medium. Postribosomal supernatants were analyzed by SDS-PAGE. A fluorogram of the region of the gel containing GRP78 is shown in the upper panel of Fig. 2. The fluorogram was densitometrically scanned and the areas were normalized to the unstarved culture. A histogram of the normalized areas is shown in the lower panel of Fig. 2. The amount of [³H]adenosine incorporated into GRP78 in 2 h is constant throughout the starvation period. Thus, even though the distribution of total GRP78 protein is established within 6 h of starvation (Fig. 1), label continues to be incorporated at a constant rate throughout the starvation period. This result indicates that the distribution of total protein between the two forms of GRP78 is due to the establishment of equal rates of ADP-ribose addition and cleavage, further supporting the notion that the reaction is readily reversible.
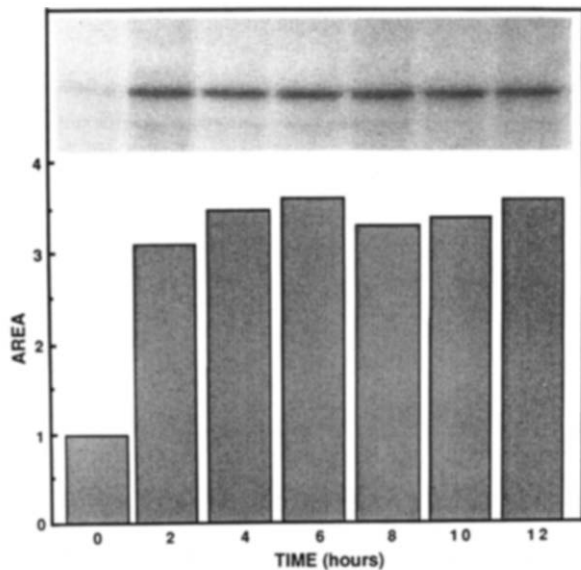
Fig. 2. ADP-ribosylation of GRP78 during amino acid starvation. Hepa cultures were fed complete medium for 2 h prior to addition of complete (control) or tryptophan-free (starvation) medium. The control culture was incubated for 2 h and the remaining cultures under starvation conditions for 2, 4, 6, 8, 10 and 12 h. During the last 2 h of the 4-12 h starvation periods, the medium was removed and the cultures were washed with Puck's saline A and refed starvation medium containing 50 μCi/ml [^{3}H]adenosine (New England Nuclear, 30 Ci/mmol). Label was added to the control and the 2 h starvation cultures immediately. Following the pulse-labeling period, cultures were harvested as previously described and postribosomal supernatants were collected and stored at $-80°$C. Total protein was determined by the method of Lowry [20]. The samples were analyzed by SDS-PAGE. Following fixation and staining, the gel was impregnated with sodium salicylate [21] and fluorographed on preflashed [22] XAR-5 film (Kodak). The fluorograph was scanned using a Joyce-Loebl densitometer equipped with an analog-to-digital converter. Peak integration was done using Vidichart (Interactive Microware, State College, PA). Areas were normalized against total protein in each sample and the area of the unstarved sample. The top panel shows the fluorogram. The lower panel shows the normalized areas as a function of starvation time.

3.3. The stress-stimulated GRP78 is available for ADP-ribosylation

GRP78 normally functions in the assembly of newly synthesized secretory proteins and integral membrane proteins [8-12]. GRP78 is overexpressed whenever aberrant proteins are produced, either by mutation or by interference with normal processing [13]. We have previously shown in Hepa cells that inhibition of N-linked glycosylation results in an accumulation of the non-ADP-ribosylated form of GRP78 suggesting that this is the functional form of the protein [16]. The aim of the following experiment was to determine if the overexpressed GRP78 was available for ADP-ribosylation. GRP78 was overexpressed by a 24 h exposure of Hepa cells to 50 nM tunicamycin in the presence of [^{3}H]leucine. Proteins from control cultures, tryptophan-starved cultures, tunicamycin treated cultures, and tunicamycin-treated starved cultures were resolved by 2-D SDS-PAGE. Individual spots corresponding to
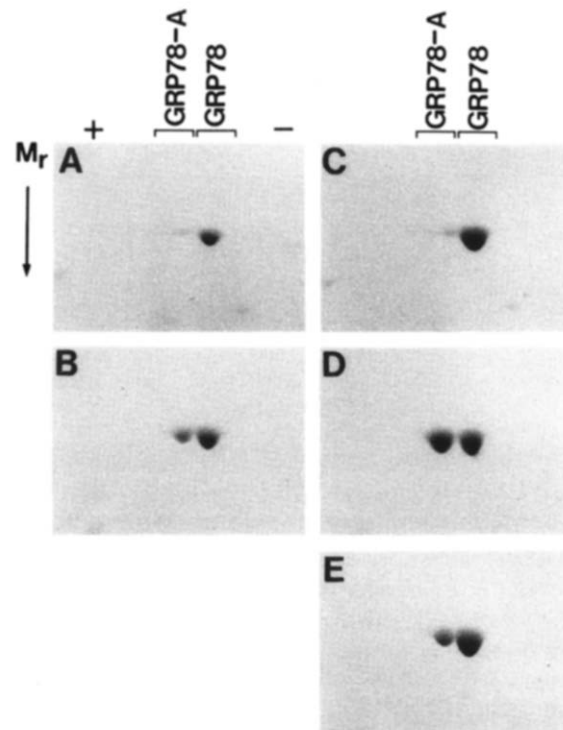

Fig. 3. Accumulation and ADP-ribosylation of GRP78 following treatment of Hepa cells with tunicamycin. Subconfluent Hepa cultures were incubated in medium containing 35 mg/1 leucine (1/3 that of complete medium) and 50 μCi/ml [^{3}H]leucine (New England Nuclear, 40-60 Ci/mmol), with or without tunicamycin (50 nM), for 24 h. Following the labeling period, all cultures were refed complete or tunicamycin-containing medium for 1 h. One culture incubated in complete medium (control) and one incubated with tunicamycin were harvested, as previously described, and the postmitochondrial supernatants stored at $-80°$C. The remaining control culture and two tunicamycin-treated cultures were refed tryptophan-free medium, one of the latter was supplemented with tunicamycin. These cultures were incubated for an additional 24 h. The cultures were then harvested as described above. Proteins were resolved by 2-D SDS-PAGE and visualized by staining with Coomassie blue. The spots corresponding to GRP78 and ADP-ribosylated GRP78 were excised, dissolved and counted as described above. Shown are the Coomassie blue stained gels in the region of GRP78 (GRP78-A is the ADP-ribosylated form). Panels A and C are from cultures harvested at the end of the labeling period. The quantitative data are in Table I. Panels B, D and E are from cultures starved of tryptophan for 24 h in the presence or absence of tunicamycin. Panel A, complete growth medium; Panel B, tryptophan-starved; Panel C, tunicamycin-treated; Panel D, tryptophan starvation following tunicamycin treatment; Panel E, tryptophan starvation in the presence of tunicamycin following the initial tunicamycin treatment.

GRP78 and ADP-ribosylated GRP78 were excised from the gels, dissolved and counted by liquid scintillation. Fig. 3 shows the Coomassie blue stained patterns; the quantitative data are in Table I. Fig. 3 (panels A and B) shows the effect of 24 h of tryptophan-starvation on the distribution of total GRP78 between the ADP-ribosylated and non-ADP-ribosylated forms. Consistent with previous results, there is a shift of protein into the ADP-ribosylated form resulting in a near-equal distribution of label between the two spots. Panel C

Table 1

Accumulation and ADP-ribosylation of GRP78 following treatment of Hepa cells with tunicamycin

Panel	Culture	(cpm)	
		GRP78	GRP78-A
A	Control	11 550	1 243
B	Trp-starvation	5 426	4 495
C	Tunicamycin	39 722	2 482
D	Trp-starvation − tunicamycin	10 436	13 117
E	Trp-starvation + tunicamycin	29 953	9 709

shows that the overexpressed GRP78 is primarily the non-ADP-ribosylated form, and Table I shows that there is approximately a 4-fold increase in the label in total GRP78 as a result of tunicamycin treatment. Panels D and E show that the GRP78 accumulated by tunicamycin treatment is available for ADP-ribosylation, particularly after tunicamycin has been removed from the culture. Additionally, comparison of the amount of label remaining in total GRP78 (panels D and E) shows that the GRP78 is stable in the presence of tunicamycin, but not if the tunicamycin is removed.

4. DISCUSSION

GRP78 has been shown to be the same as BiP [23,24] which participates in the assembly of immunoglobulin IgG [25]. GRP78 binds to the heavy chain and retains it in the lumen of the endoplasmic reticulum until the light chain is associated with it. Hendershot et al. [24] have shown that when the immunoglobulin heavy chain and GRP78 are co-immunoprecipitated, there is no ADP-ribose associated with GRP78 indicating that the functional form of GRP78 is non-ADP-ribosylated. This is supported by the demonstration that the GRP78 overexpressed during tunicamycin treatment is almost exclusively the non-ADP-ribosylated form [16].

The data presented in this report illustrate two important points. First, the over-expressed GRP78 does not represent a distinct pool of GRP78 unavailable for modification by ADP-ribosylation, and second, the stability of the induced pool of GRP78 as well as the extent of its ADP-ribosylation is dependent upon whether inhibition of glycosylation is maintained during nutritional stress. It is possible that the ADP-ribosylation of GRP78 may be restricted, in part, in the presence of tunicamycin through the association of GRP78 with aberrant, under-glycosylated proteins. In theory, this could be due either to interference with the site of ADP-ribosylation by its association with misfolded proteins or to a more general influence of tunicamycin on cellular metabolism.

The data presented in this report also illustrate several important features of the ADP-ribosylation of GRP78. As previously shown, the majority of GRP78 is non-ADP-ribosylated under fed conditions, while during amino acid starvation, GRP78 becomes ADP-ribosylated. Here we have shown that by approximately 6 h of amino acid starvation, there is no longer a net gain in [^{35}S]methionine incorporation into the ADP-ribosylated form of GRP78 (Fig. 1). However, throughout the 12 h period of starvation the extent of ADP-ribosylation of GRP78 is constant within any 2 h period (Fig. 2). Taken together, these data suggest that the rate of ADP-ribosylation of GRP78 must be greater than the rate of cleavage of the ADP-ribose from GRP78 during the early period of starvation resulting in an accumulation of this form. However, the rate of addition and cleavage of ADP-ribose must be equal during prolonged starvation. Such a situation would result in the maintenance of a non-ADP-ribosylated pool of GRP78 which may be critical for the interaction of GRP78 with aberrant proteins as well as in the assembly of normal secretory and integral membrane proteins.

Clear precedence for metabolic control by reversible ADP-ribosylation has been established in nitrogen fixing bacteria. Nitrogen fixation consumes up to 30 molecules of ATP per molecule of nitrogen converted to ammonia. The existence of fixed nitrogen [5,6] in the environment of the bacteria activates an ADP-ribosyltransferase which inhibits nitrogen fixation by ADP-ribosylation of dinitrogenase reductase, and thereby spares the unnecessary expenditure of energy reserves. The enzyme is reactivated by a glycohydrolase (dinitrogenase reductase activating glycohydrolase) which cleaves the N-glycosidic linkage between arginine and ADP-ribose [26].

There is other evidence supporting the existence of reversible ADP-ribosylation reactions in higher eukaryotes. A glycohydrolase which cleaves arginine-(ADP-ribose linkages) has been identified in [27] and purified from turkey erythrocytes [28]. Further, elongation factor 2, EF-2, has been shown to be ADP-ribosylated by an endogenous transferase during serum-starvation; and that the reaction is reversible upon addition of serum [29]. Diphtheria toxin inhibits protein synthesis by ADP-ribosylation of EF-2 [30]. It then follows that reduced rates of translation during serum starvation are the result of ADP-ribosylation of EF-2. It has previously been shown that inhibition of protein synthesis results in the ADP-ribosylation of GRP78. Thus serum-starvation would be predicted to result in a cascade of ADP-ribosylation reactions.

The ADP-ribosylation of GRP78 is an extremely specific event. Resolution of cellular proteins by 2-D electrophoresis has shown that under the electrophoretic conditions used, GRP78 appears to be the only protein so modified. The physiological significance of this process is yet to be determined. It seems clear, however, that the extent of ADP-ribosylation is coupled to the translational activity of the cells and may

be part of a nutritional stress response. The demonstration that the process is reversible suggests that ADP-ribosylation of GRP78 may be part of a metabolic control mechanism.

REFERENCES

[1] Ui, M. (1984) Trends Pharmacol. Sci. 5, 277–279.
[2] Vaughan, M. and Moss, J. (1982) Curr. Top. Cell. Regul. 20, 205–246.
[3] Tanuma, S.-I., Kawashima, K. and Endo, H. (1988) J. Biol. Chem. 263, 5485–5489.
[4] Lowery, R.G., Saari, L.L. and Ludden, P.W. (1986) J. Bacteriol. 166, 513–518.
[5] Fu, H., Burris, R.H. and Roberts, G.P. (1990) Proc. Natl. Acad. Sci. USA 87, 1720–1724.
[6] Kanemoto, R.H. and Ludden, P.W. (1987) J. Bacteriol. 169, 3035–3043.
[7] Pope, M.R., Murrell, S.A. and Ludden, P.W. (1985) Proc. Natl. Acad. Sci. USA 82, 3173–3177.
[8] Pelham, H.R.B. (1986) Cell 46, 959–961.
[9] Schlesinger, M.J. (1986) J. Cell Biol. 103, 321–325.
[10] Gething, M.-J., McCammon, K. and Sambrook, J. (1986) Cell 46, 939–950.
[11] Ellis, R.J. and Hemmingsen, S.M. (1989) Trends Biochem. Sci. 14, 339–342.
[12] Rothman, J.E. (1989) Cell 59, 591–601.
[13] Lee, A.S. (1987) Trends Biochem. Sci. 12, 20–23.
[14] Carlsson, L. and Lazarides, E. (1983) Proc. Natl. Acad. Sci. USA 80, 4664–4668.
[15] Ledford, B.E. and Jacobs, D.F. (1986) Eur. J. Biochem. 161, 661–667.
[16] Leno, G.H. and Ledford, B.E. (1989) Eur. J. Biochem. 186, 205–211.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[19] Donato, H., Doig, M.T. and Priest, D.G. (1988) J. Biochem. Biophys. Methods 15, 331–336.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.
[22] Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335–341.
[23] Munro, S. and Pelham, H.R.B. (1986) Cell 46, 291–300.
[24] Hendershot, L.M., Ting, J. and Lee, A.S. (1988) Mol. Cell. Biol. 8, 4250–4256.
[25] Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) J. Cell Biol. 102, 1558–1566.
[26] Saari, L.L., Pope, M.R., Murrell, S.A. and Ludden, P.W. (1986) J. Biol. Chem. 261, 4973–4977.
[27] Moss, J., Jacobson, M.K. and Stanley, S.J. (1985) Proc. Natl. Acad. Sci. USA 82, 5603–5607.
[28] Moss, J., Tsai, S.-C., Adamik, R., Chen, H.-C. and Stanley, S.J. (1988) Biochemistry 27, 5819–5823.
[29] Fendrick, J.L. and Iglewski, W.J. (1989) Proc. Natl. Acad. Sci. USA 86, 554–557.
[30] Collier, R.J. (1982) in: ADP-ribosylation reactions (Haysishai, O. and Ueda, K., eds) pp. 575–592, Academic Press, New York.