Evidence of a role for NAD⁺-glycohydrolase and ADP-ribosyltransferase in growth and differentiation of *Streptomyces griseus* NRRL B-2682: inhibition by *m*-aminophenylboronic acid

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***m*-Aminophenylboronic acid (APBA) inhibited the germination, growth and sporulation of *Streptomyces griseus* NRRL B-2682 in an age- and concentration-dependent manner in submerged and solid cultures. When added to cells or cell extracts it irreversibly inhibited NAD⁺-glycohydrolase and ADP-ribosyltransferase activity. ADP-ribosyltransferase was more sensitive, but inhibition was not complete, even in the presence of 10 mM APBA. The *in vivo* effects of the inhibitor correlated with its *in vitro* effect on ADP-ribosylation and on the profile of ADP-ribosylated endogenous proteins. The physiological importance of ADP-ribosyltransferase was supported by the observation that APBA strongly inhibited the growth of a non-sporulating and NAD⁺-glycohydrolase-negative mutant of the parental strain. The resistance of *S. griseus* NRRL B-2682 strains able to grow in the presence of APBA was due to permeability factors. A comparison of the ADP-ribosylated protein profiles of *S. griseus* NRRL B-2682 grown under various conditions showed similarities, but also specific differences. The results suggest that the ADP-ribosyltransferase of *S. griseus* NRRL B-2682 is an indispensable enzyme for growth and differentiation of the strain. It may regulate the activity of key enzymes or developmental proteins by responding to intra- and extracellular conditions.**

Keywords: *Streptomyces griseus*, ADP-ribosyltransferase, NAD⁺-glycohydrolase, *m*-aminophenylboronic acid, sporulation

INTRODUCTION

Streptomyces are filamentous Gram-positive soil bacteria, well known for their complex morphological development and their ability to produce a wide variety of secondary metabolites. On solid media the spores germinate to produce a network of apically growing hyphae that form the substrate mycelium. This later develops into aerial mycelium, which eventually produces terminally differentiated spore cells. Certain species can sporulate in submerged cultures, although here the substrate mycelium develops into spores without forming aerial mycelium. The early events in sporulation often coincide

with the initiation of secondary metabolite production. The genetics of the developmental process have been extensively analysed, especially in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus* (Chater, 1984; Babcock & Kendrick, 1988; Ensign, 1988; Chater, 1993). In the latter organism cell differentiation and secondary metabolite production are initiated by A-factor, a hydrophobic γ -butyrolactone (Khokhlov *et al.*, 1973; Miyake *et al.*, 1990; Horinouchi & Beppu, 1992).

Some biochemical pathways are regulated post-translationally by covalent modification of proteins through phosphorylation, adenylation, methylation or poly- or mono-ADP-ribosylation (Streicher & Tyler, 1981; Nimmo, 1987; Fu *et al.*, 1990; Lowery & Ludden, 1990; Hong *et al.*, 1991; Hoch, 1993; Morgan *et al.*, 1993). Mono-ADP-ribosylation reactions are catalysed by mono-

Abbreviations: ADPRTase, ADP-ribosyltransferase; APBA, *m*-aminophenylboronic acid; NAD⁺ase, NAD⁺-glycohydrolase.

ADP-ribosyltransferases (ADPRTases); these transfer the ADP-ribose moiety of NAD⁺ to acceptor proteins, which are thereby altered in functional properties (Hayashi & Ueda, 1985). The best studied bacterial mono-ADPRTases are bacterial toxins like those of cholera, pertussis or botulinum C3, which have as a common feature the ADP-ribosylation of eukaryotic GTP-binding proteins (Chung & Collier, 1977; Ui, 1990; Iiri *et al.*, 1992; Machama *et al.*, 1994; Spangler, 1992). Endogenous mono-ADP-ribosylation has been demonstrated in several bacteria; however, except in certain photosynthetic bacteria, little is known about the physiological role of this protein modification process (Edmonds *et al.*, 1989; Fu *et al.*, 1989, 1990; Lowery & Ludden, 1990; Penyige *et al.*, 1990; Eastman & Dworkin, 1994).

A regulatory role for ADP-ribosylation in prokaryotes has been established in *Rhodospirillum rubrum* where it controls the activity of the nitrogenase complex. The inactive ADP-ribosylated form of the enzyme is reactivated by mono-ADP-ribose glycohydrolase-catalysed removal of the ADP-ribose group. The activities of the modifying enzymes are regulated by metabolic factors (Fu *et al.*, 1990; Lowery & Ludden, 1990). Glutamine synthetase is also ADP-ribosylated in *R. rubrum*, *S. griseus* NRRL B-2682 and in *Synechocystis* species (Woehle *et al.*, 1993; Penyige *et al.*, 1994; Silman *et al.*, 1995). The ADP-ribosylated protein profile of *S. griseus* shows differentiation stage-specific changes, and inhibition of ADPRTase during the early exponential growth phase decreases sporulation (Ochi *et al.*, 1992; Penyige *et al.*, 1992).

m-Aminophenylboronic acid (APBA) is a well known inhibitor of sporulation in bacilli, and in *Bacillus sphaericus* it inhibits both sporulation and chemotactic behaviour without inhibiting growth (Andrew *et al.*, 1994). To have this effect, APBA must be added before the onset of late exponential growth. APBA may inhibit a regulatory pathway common to sporulation and chemotaxis and as a result inhibit methyl group turnover in the methyl-accepting protein of *B. sphaericus* (Andrew *et al.*, 1994). However, the molecular target of APBA is not known. Inhibition of proteases has been suggested in bacilli but some protease-negative mutants are as sensitive as the wild-type strain (Andrew *et al.*, 1994).

We report here the *in vivo* and *in vitro* effect of APBA on another Gram-positive soil bacterium. *S. griseus* NRRL B-2682 is more sensitive than *B. sphaericus* and analysis of its effects has indicated that its ADP-ribosylation targets are important in regulating growth and differentiation.

METHODS

Bacterial strains and culture conditions. The wild-type streptomycin producer *Streptomyces griseus* NRRL B-2682 was a generous gift from J. C. Ensign, Dept. of Bacteriology, University of Wisconsin-Madison, USA. Strain B-2682-3 was a newly isolated non-sporulating spontaneous mutant that was A-factor responsive (A-factor restored spore formation). Cells were grown in: (i) defined minimal (DM) medium containing 25 mM MOPS/KOH, pH 7.2, 50 mM glucose, 5 mM NH₄Cl, 0.5 mM MgCl₂, 10 mM KH₂PO₄/K₂HPO₄, 0.05 % casamino

acids and 0.4 % trace salt solution (TS: 17 mM CaCl₂, 5 mM FeSO₄, 20 mM MnSO₄, 0.3 mM ZnSO₄); (ii) GYM medium containing 0.4 % glucose, 0.4 % yeast extract, 1.0 % (w/v) malt extract, 0.1 % NZ amine, 0.2 % NaCl and 0.3 % TS; (iii) SPY medium containing 3 % (w/v) soluble starch, 0.2 % yeast extract, 3 % (w/v) polypeptone, 0.2 % MgSO₄ and 0.3 % TS. Media (100 ml) were inoculated with 300 µl spore stock suspension and cultures were incubated in 500 ml Erlenmeyer flasks at 27 °C in a gyratory shaker at 250 r.p.m. Solid cultures were grown at 27 °C on DM-agar (DM containing 2 %, w/v, Bacto-agar). Spore stock suspension (100 µl) was spread on the agar surface. Spores of the Spo⁻ mutant were produced on DM-agar supplemented with 3 × 10⁻⁷ M A-factor, and this spore stock suspension was used to inoculate all Spo⁻ cultures. In inhibitor studies with submerged cultures, APBA was added as a sterile-filtered aqueous solution at the appropriate times; with solid cultures Whatman 3 MM paper discs supplemented with APBA solutions were placed on the agar surface.

Preparation of cell extracts for enzyme assays. Mycelium was harvested from cultures in mid-exponential growth by centrifugation at 6000 *g* and 4 °C for 20 min. The growth phase of cultures was determined by measuring the increase in protein content of samples taken at 30 min intervals. The mycelium was washed twice and resuspended in 25 mM Tris/HCl buffer, pH 7.2, containing 0.5 mM PMSF. Cells were disrupted by sonication for 2 min at 0 °C using a Branson Model 250 Sonifier. The homogenate was centrifuged at 10000 *g* for 20 min at 4 °C, and the supernatant was used as cell extract for enzyme assays. When the *in vivo* effect of APBA was determined, cultures were supplemented with 50 µM APBA; preparation of these crude extracts was the same as above except that the harvested mycelium was washed five times.

In vitro detection of endogenous ADP-ribosylated proteins.

Crude cell extracts containing 100 µg protein were resuspended in ADP-ribosylation buffer containing 20 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 20 µM ADP-ribose and 1 µM [³²P]NAD⁺ (1.85 × 10⁵ Bq) to give 100 µl final volume. After 20 min incubation at 32 °C, 100 µl ice cold 20 % (w/v) trichloroacetic acid (TCA) was added and the proteins precipitated at 0 °C for 30 min were collected by centrifugation (12000 *g* for 5 min). The pellet was washed twice with 10 % (w/v) TCA, then with diethyl ether. It was solubilized overnight at 4 °C in 40 µl concentrated (2×) electrophoresis sample buffer (Laemmli, 1970) mixed with 5 µl 10 % (w/v) SDS. Before electrophoresis, samples were heated at 80 °C for 4 min. ADP-ribosylated proteins were separated on 13 % (w/v) SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue, destained and dried; labelled proteins were detected by autoradiography (5 d exposure to X-ray film at -80 °C with an intensifying screen).

ADPRTase and NAD⁺-glycohydrolase (NAD⁺ase) assays.

Incorporation of ADP-ribose into proteins of crude cell extracts was measured in a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 20 µM ADP-ribose, 2 µM [2,8-adenine-³H]NAD⁺ (5 × 10⁵ d.p.m. per sample) and 50 µg protein in a final volume of 50 µl with or without the appropriate amount of APBA. Reactions were terminated after 20 min incubation at 32 °C by adding 50 µl 20 % (w/v) TCA. Proteins precipitated after 30 min at 0 °C were collected by filtration through nitrocellulose discs (Millipore; 45 µm pore size) and washed three times with 5 ml ice cold TCA and once with diethyl ether. Radioactivity on the filters was determined by liquid scintillation counting.

NAD⁺ase activity was determined as described in the Worthington Enzyme Manual (Worthington, 1972). Crude

cell extracts were added to a buffer containing 100 mM K_2HPO_4/KH_2PO_4 , pH 7.2, and 0.2 mM NAD^+ with or without APBA; the final volume was 500 μ l. After incubation at 32 °C for 5 min, 500 μ l 1 M KCN was added and after another 5 min the absorbance of the samples at 340 nm was determined. In control samples the KCN solution was added before incubation. NAD^+ ase activity was calculated according to Worthington (1972). In inhibitor assays crude extract was pre-incubated with APBA for 5 min before the reaction was started by addition of the substrate. When the reversibility of APBA inhibition was studied, crude extract that had been concentrated by ultrafiltration using Centricon-10 units (Amicon) were pre-incubated for 5 min with APBA at the appropriate temperature, then diluted 100 times. One unit of enzyme activity cleaves 1 μ mol NAD^+ min^{-1} at 32 °C.

Partial purification of NAD^+ ase. Crude extract preparations of *S. griseus* NRRL B-2682 mycelium were applied to an Affi-Gel Blue (Bio-Rad) column (1.5 $\times$ 9 cm), previously equilibrated with 25 mM Tris/HCl, pH 7.2 (buffer A). The column was washed with 5 vols buffer A and the enzyme was eluted with 0.9 M NaCl in buffer A. Active fractions were concentrated and introduced onto a Reactive Red (Sigma) column (1 $\times$ 5 cm) equilibrated with 0.9 M NaCl in buffer A. The column was washed with 5 vols 2 M NaCl in buffer A; the NAD^+ ase was then eluted with 3 M NaCl in buffer A. The samples were concentrated and used as partially purified enzyme in further experiments. Fractions were assayed for NAD^+ ase as described above.

RESULTS

Effect of APBA on growth and differentiation of *S. griseus* NRRL B-2682

On DM-agar, *S. griseus* NRRL B-2682 cultures formed aerial mycelium by 24 h after inoculation and started to sporulate at 36 h. Since its effect was growth phase-specific in bacilli, APBA was added to cultures at two different times. Previously it was suggested that *S. griseus* NRRL B-2682 becomes committed to sporulation after growth for 16–18 h on DM medium (Ensign, 1988). Therefore paper discs containing 2 μ mol APBA were deposited on the plates at inoculation and at 24 h (after the commitment time). The effect of APBA is shown in Fig. 1 and was confirmed by phase-contrast microscopy (data not shown). When added at 0 time, APBA strongly inhibited spore germination and the outgrowth of germ tubes and hyphae (large clear halo around the disc). Dilution of the inhibitor during diffusion resulted in an outer gray zone where growth occurred but aerial mycelium and spores were not formed. Outside this was an area where aerial mycelium was present but spore formation was still inhibited (the edge of the white area around the gray halo). The same amount of APBA added at 24 h was less inhibitory; however, the inhibition zone around the disc showed that APBA caused cell lysis (Fig. 1). Aerial mycelium and spore formation were also somewhat inhibited at lower APBA concentrations around the disc. After 2 d of incubation, numerous small, APBA-resistant colonies appeared in the previously clear inhibition zone. Five of these spontaneous mutants were isolated and analysed as described below.

The effect of APBA on the non-sporulating NAD^+ ase-

Fig. 1. Inhibition of growth, aerial mycelium and spore formation of *S. griseus* NRRL B-2682 by APBA. The cells were grown on DM-agar at 27 °C. Paper discs containing 2 μ mol APBA were placed on the surface of the culture at the time of inoculation (on the right of the Petri dish, a large clear halo is visible around this disc) and at 24 h after inoculation (on the left of the dish). The photograph was taken at 48 h.

Fig. 2. Phase contrast photomicrograph taken 24 h after inoculation showing the effect of APBA on germination and growth of submerged cultures of *S. griseus* NRRL B-2682 in DM medium. APBA (60 μ M) was added at the time of inoculation. Bar, 20 μ m.

negative *S. griseus* mutant B-2682-3 was basically the same as for the parent: when added at 0 time it strongly inhibited germination and growth of the strain (data not shown).

For cultures grown in liquid DM medium, the effect of APBA was both concentration- and growth phase-dependent. The MIC_{50} of APBA added at 0 time was 40 μ M, based on measuring growth as the increase in protein content of cultures (data not shown). Addition of 60 μ M APBA at inoculation severely inhibited germination and delayed hyphal growth (Fig. 2). Even 24 h

Fig. 3. Phase contrast photomicrographs taken at 48 h showing the effect of 15 μ M APBA on the differentiation of submerged cultures of *S. griseus* NRRL B-2682 in DM medium. The inhibitor was added at inoculation. (a) APBA-inhibited culture (no sporulation); (b) control culture (abundant sporulation). Bars, 20 μ m.

after inoculation, when the control culture contained well developed mycelium, only germ tubes and non-germinated spores were present in the APBA-treated culture. At 15 μ M, APBA reduced growth by only 10%, but inhibited sporulation completely (Fig. 3). Adding 40 μ M APBA 24 h after inoculation induced strong lysis of the cells (data not shown).

Inhibition of NAD⁺ase and ADPRTase activity by APBA in cell extracts of *S. griseus* NRRL B-2682 and its APBA-resistant mutant

To determine the possible cellular targets of APBA we examined its effect on NAD⁺ase and ADPRTase activities in crude extracts prepared from *S. griseus* NRRL B-2682 cells grown for 16 h in DM medium. As reported previously (Penyige *et al.*, 1992), *S. griseus* NRRL B-2682 cells contained both enzymes. The former belongs to the nicotinamide-insensitive class of NAD⁺ases while the latter is an arginine-specific mono-ADPRTase (Oppenheimer & Handlon, 1992; Penyige *et al.*, 1990). From

Fig. 4. Effect of APBA on the NAD⁺ase (a) and ADPRTase (b) activity. (a) NAD⁺ase in a crude extract of *S. griseus* NRRL B-2682 cells (□), a partially purified extract of *S. griseus* NRRL B-2682 (▨) and a crude extract of the APBA-resistant mutant (■) incubated with varying concentrations of APBA. NAD⁺ase activity was monitored as described in Methods. The specific activity of NAD⁺ase was 90.63 U (mg protein)⁻¹ min⁻¹ in the crude cell extract of parental *S. griseus* NRRL B-2682. (b) Incorporation of [³²P]ADP-ribose into proteins in a crude extract of *S. griseus* NRRL B-2682 cells incubated with [³²P]NAD⁺ in the presence of various concentrations of APBA. The procedure is described in Methods. All values are means of triplicate determinations. The $\pm$ SE values were always less than 6 U in all NAD⁺ase measurements and 500 d.p.m. in the ADPRTase measurements.

crude cell extracts of *S. griseus* NRRL B-2682, a partially purified NAD⁺ase was isolated.

Concentrations of APBA in the 0.05–20 mM range caused a gradual, almost complete, concentration-dependent inhibition of the NAD⁺ase (Fig. 4a). In the crude extract APBA effectively inhibited the enzyme with an IC₅₀ of about 6.6 mM. With the partially purified enzyme, the IC₅₀ was 3.4 mM (Fig. 4a). In comparison, 20 mM nicotinamide, a competitive analogue of NAD⁺, caused

Fig. 5. (a) *In vitro* inhibition of endogenous ADP-ribosylation with APBA. Proteins in crude cell extracts from *S. griseus* NRRL B-2682 grown in DM medium (lanes 1 and 2), GYM medium (lanes 3 and 4) and SPY medium (lanes 5 and 6) were ADP-ribosylated in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 50 μ M APBA using [32 P]NAD $^{+}$ as the substrate. The samples were subjected to SDS-PAGE and ADP-ribosylated proteins were detected by autoradiography. (b) *In vivo* inhibition of endogenous ADPRTase of *S. griseus* NRRL B-2682 with APBA. Crude cell extracts were prepared from 16-h-old mycelium grown in DM liquid medium in the presence or absence of 50 μ M APBA. Proteins from the crude extracts were incubated with [32 P]NAD $^{+}$ as described in Methods. Labelled proteins were separated by SDS-PAGE and detected by autoradiography. Lanes: 1, control culture; 2, APBA-treated culture.

only 1% inhibition of the partially purified NAD $^{+}$ ase, while 3-aminobenzamide – also an analogue – was even less effective (data not shown) when tested under these conditions. The NAD $^{+}$ ase activity in cell extracts of all five APBA-resistant mutants showed similar sensitivity toward the inhibitor (Fig. 4a; only one representative sample is shown). The concentration-dependent manner in which APBA inhibited the NAD $^{+}$ ase of these strains suggested that the inhibitor was not inactivated. Therefore, the resistance of these strains may have been due to permeability factors. The mutants differ from the wild-type in several characteristics: they form aerial mycelium and spores 8–10 h earlier than *S. griseus* NRRL B-2682 in solid DM medium and produce less than one-third, on average, of the parental levels of streptomycin.

APBA was more inhibitory towards the endogenous ADPRTase (Fig. 4b) but still caused only limited inhibition. The IC_{50} of APBA was about 0.8 mM in the crude extract, higher concentrations augmented the inhibition and at 10 mM it was 78%. In the range of 0.05–1 mM, APBA caused a steep drop in ADPRTase activity but increasing the APBA concentration to 10 mM resulted in only 20% additional inhibition. Moreover, there remained a further 22% residual ADPRTase activity.

To examine further the reversibility of APBA inhibition of NAD $^{+}$ ase and ADPRTase activities, the crude extract of *S. griseus* NRRL B-2682 cells was preincubated with 10 mM APBA for 5 min, then diluted 100 times and the specific activities of the enzymes redetermined. Dilution did not diminish the inhibitory effect of APBA. The specific activity of the NAD $^{+}$ ase was only 20% of the control sample. The value was 18% in the case of the ADPRTase (data not shown). These values agree well with previous measurements (Fig. 4a and b).

The effect of APBA on the ADP-ribosylated protein profile of *S. griseus* NRRL B-2682

Since *in vivo* labelling of proteins with [32 P]NAD $^{+}$ was not possible due to permeability problems, ADP-ribosylation was examined in cell extracts. These were prepared from *S. griseus* NRRL B-2682 cells grown for 16 h in DM, GYM and SPY liquid media. Proteins were labelled by incubating extracts with [32 P]NAD $^{+}$ in the absence or presence of 50 μ M APBA and the reaction products separated by SDS-PAGE were detected by autoradiography. In the extract from DM-grown cells, five major proteins with molecular masses of 59, 55, 50, 43 and 41 kDa and three minor ones (36, 21 and 16 kDa) were ADP-ribosylated (Fig. 5a, lane 1). APBA at 50 μ M almost completely inhibited the labelling of all except the 43 kDa protein, which was affected but to a lesser extent (Fig. 5a, lane 2). Four major ADP-ribosylated proteins (81, 59, 50 and 43 kDa) and four additional minor ones (36, 31, 26 and 21 kDa) were present in the crude extract of GYM-grown cells (Fig. 5a, lane 3). Again APBA almost completely inhibited ADP-ribosylation of all except the 81 and 43 kDa proteins. Surprisingly, a 16 kDa ADP-ribosylated band not present in the control lane was

visible in this sample (Fig. 5a, lane 4). In crude extracts of SPY-grown cells, the major ADP-ribosylated protein bands were at 59, 55, 50 and 43 kDa and the minor ones were at 36, 26 and 21 kDa (Fig. 5a, lane 5). The inhibitory effect of APBA seemed to be strongest in this extract, since only weakly labelled bands at 43 and 16 kDa were visible (Fig. 5a, lane 6). As before, a 16 kDa protein was labelled only in the presence of APBA.

In all three APBA-inhibited samples, a small (8–10 kDa) ADP-ribosylated peptide was visible on the gel just above the dye front. This small peptide was not or was only very weakly labelled in the control samples and the intensity of the band varied in different samples. The presence of weakly labelled proteins might account for the residual ADPRTase activity found earlier with 10 mM APBA. The ADP-ribosylated protein profile and the intensity of label in certain proteins differed in the extracts from cells grown in different media. Some proteins, e.g. those at 59, 50, 43 and 36 kDa were ADP-ribosylated in all three control samples, albeit at different intensities of label. Other proteins, or their ADP-ribosylation, were confined to extracts from cells grown in certain media. Although the profile of ADP-ribosylated proteins evidently varied with the growth conditions, APBA was inhibitory in all three samples examined.

The *in vivo* inhibitory effect of APBA

APBA at 50 μ M was added to submerged cultures grown for 4 h in DM medium. As before, growth and differentiation of the cells were strongly inhibited (data not shown). After 16 h the cells were harvested and washed to remove the inhibitor. The specific activity of NAD⁺ase in crude cell extracts of the APBA-inhibited culture was 0.40 U (mg protein)⁻¹ ml⁻¹, only 0.5% of that in crude cell extracts from control cultures, which averaged 85 U (mg protein)⁻¹ ml⁻¹. The ADPRTase activity was similarly reduced. Electrophoresis and autoradiography to detect ADP-ribosylated proteins showed much weaker labelling in this sample than in the control (Fig. 5b, lanes 2 and 1, respectively). Since thorough washing of the mycelium and dilution of the samples during crude extract preparation did not relieve the inhibitory effect of APBA, the results indicate that APBA irreversibly inhibited the enzymes, although the less likely possibility that APBA inhibited their synthesis was not excluded.

DISCUSSION

We have previously proposed that endogenous ADP-ribosylation of proteins is involved in regulating sporulation and antibiotic production in *S. griseus* (Ochi *et al.*, 1992; Penyige *et al.*, 1992). These proposals were based primarily on the effect of ADPRTase inhibitors on differentiation, on changes in the ADP-ribosylated protein profile during the life cycle of *S. griseus* strains and on differences in this profile between the wild-type and its non-sporulating mutants. The results presented here further support our previous observations in suggesting that growth and differentiation in *S. griseus* NRRL B-2682 require ADPRTase activity.

Our results indicate that *S. griseus* NRRL B-2682 is more sensitive to APBA than *B. spheericus*. Addition of APBA to *S. griseus* NRRL B-2682 cultures grown on DM-agar resulted in a concentration- and age-dependent inhibition of germination and mycelial growth. APBA had less effect when added in the late exponential growth phase. In the agar-diffusion assay the concentrations of APBA that permitted growth still inhibited aerial mycelium and spore formation. The growth of a Spo⁻ mutant of *S. griseus* NRRL B-2682 was as sensitive to APBA as the parental strain. In submerged cultures APBA effectively inhibited germination, growth and sporulation of *S. griseus* NRRL B-2682 in a concentration-dependent manner when added before the late exponential growth phase; later addition caused lysis of the mycelium.

APBA readily interacts with glycoproteins containing vicinal diol groups in their carbohydrate moiety and is frequently used to isolate glycosylated proteins (Bisse & Wieland, 1992; Jobst *et al.*, 1992). Glycoproteins are rare in prokaryotes, but there are indications that the NAD⁺ase of *S. griseus* NRRL B-2682 is glycosylated (A. Bertinusson & J. C. Ensign, personal communication).

Since the effects of APBA in *S. griseus* NRRL B-2682 agreed well with the effects of ADPRTase inhibitors we have observed previously (Penyige *et al.*, 1992), we tested the effect of APBA on the NAD⁺ase and ADPRTase present in a cell extract of *S. griseus* NRRL B-2682. It is still not clear whether or not different proteins are responsible for these enzyme activities because several ADPRTases have NAD⁺ase activity too (Hayashi & Ueda, 1985; Oppenheimer & Handlon, 1992). One might expect similarities between these enzymes, since the glycohydrolytic step is common in both enzyme reactions. Preincubation of crude extracts with APBA inhibited both enzyme activities, with the degree of inhibition being concentration-dependent. ADPRTase was the more sensitive, especially at lower APBA concentrations (0.05–1 mM), but even 10 mM APBA did not completely inhibit the enzyme. Residual ADPRTase activity was also evident on autoradiograms of ADP-ribosylated proteins, which showed that although 50 μ M APBA severely inhibited ADP-ribose incorporation, some of the proteins continued to be ADP-ribosylated, albeit to a lesser extent. A possible explanation could be that the affinity of the enzyme for certain proteins is affected differently by binding of the inhibitor or, alternatively, that APBA-sensitive and -insensitive ADPRTases are present in *S. griseus* NRRL B-2682. More than one ADPRTase enzyme has been found in some other organisms (Lowery & Ludden, 1990; Woehle *et al.*, 1993).

Unlike nicotinamide and 3-aminobenzamide, APBA inhibited NAD⁺ase even at low concentrations (0.05–1 mM) and 20 mM completely abolished activity in the crude extract. The partially purified NAD⁺ase was similarly inhibited by APBA and its lower sensitivity in the crude extract might be explained by the presence of compounds like nucleotides or carbohydrates that could bind the inhibitor, thus reducing its effective concentration (which also implies that the IC₅₀ of APBA toward these enzymes may be lower than the measured values).

Both *in vivo* and *in vitro*, APBA irreversibly inhibited both the NAD⁺ase and ADPRTase. However, *in vivo* the cell growth, differentiation and even the intracellular NAD⁺ase and ADPRTase were inhibited by somewhat lower concentrations than *in vitro*. This cannot be explained without knowing the mechanism of uptake and intracellular concentration of APBA in intact cells.

The APBA sensitivity of NAD⁺ase and ADPRTase in APBA-resistant mutants provided evidence that the resistance of these strains could be due to permeability factors. The severe inhibitory effect of APBA on the growth and sporulation of *S. griseus* NRRL B-2682 and on the growth of its Spo⁻ and NAD⁺ase-negative mutant suggests that ADPRTase may regulate indispensable biochemical processes of *S. griseus* NRRL B-2682 by modulating the activity of their enzyme(s) through ADP-ribosylation. This notion is supported by the comparison of ADP-ribosylated protein profiles of cells grown under different culture conditions. Considered with our previous results (Penyige *et al.*, 1992), those reported here allow us to conclude that ADPRTase has a key function in regulating growth and differentiation in *S. griseus* NRRL B-2682.

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