PURIFICATION AND SPECTROPHOTOMETRIC ASSAY OF NEOMYCIN PHOSPHOTRANSFERASE II[1]

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SUMMARY: Neomycin phosphotronnferase II is maximally released by osmatic shocking of R+ E. coli between late log and early stationary phase. A 300-400-fold purification of the enzyme protein is accomplished by streptomycin sulfate and ammonium sulfate precipitations of osmotic shockates, followed by affinity and ion-exchange chromatography. The recovered enzyme preparation is electrophoretically 90% pure, is free of ATP-ase activity, and can be conveniently assayed spectrophotometrically by linking the production of ADP to pyruvate kinase and lactate dehydrogenase. The purified enzyme, however, is not stable.

INTRODUCTION

A new family of enzymes, found in gram negative bacteria carrying R-factors, inactivate aminoglycoside antibiotics and act as the principle agents of clinical resistance to antibiotic therapy (1). Investigations of the characteristics of these enzymes have been hindered by a lack of highly purified preparations and reliable assay methods. ATP-dependent phosphorylation is a common enzymatic mechanism of inactivation by R^+ bacteria. Consequently, some of the inactivating enzymes resemble the kinase enzymes and, once purified, should be subject to similar methods of assay and study.

Neomycin phosphotransferase II catalyzes an ATP- dependent phosphorylation of the 3'hydroxy group of the aminohexose

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moiety of kanamycins, neomycins, paromomycins, ribostamycin, and butirosins (2-4). The initial discuvery, partial characterization, and partial purification (< 10% purity) relied upon fixed time assays employing radioactivity (2) or microbiological activity (4) measurements. This report describes a highly purified preparation conveniently assayed by the continuous spectrophotometric method employed for other kinase enzymes.

EXPERIMENTAL

Kanamycin sulfate (potency 750 meq/mg) and E. coli JR76.2/W677[3] were gifts from Dr. Julian Davies. The organism produces gentamicin adenylyl transferase, described previously (5), as well as neomycin phosphotransferase II. The procedures for growing the bacteria, extracting enzymes by osmotic shocking, precipitating proteins with ammonium sulfate, and chromotographing the recovered protein on DEAE agorose were identical for both enzymes. Column fractions containing neomycin phosphotransferase II were desalted on a column of Bio-Gel P-2, equilibrated with 10 mM tris-HCl buffer pH 7.8 containing 0.125 mM EDTA, 0.3 mM dithiothreitol, and 1 mM Mg++ (standard buffer). The desalted enzyme was applied to a gentamicin C_{1a}-Affi-Gel 10 affinity column described previously (6), and eluted with 2M ammonium sulfate in standard buffer. Peak fractions were again desalted on Bio-Gel P-2 and applied to a second column of DEAE agarose (Bio Rad, DEAE Bio-Gel A) equilibrated with standard buffer. The purified enzyme was eluted from DEAE agarose with a 0-0.3 M potassium acetate gradient in standard buffer.

Column elution gradients were determined from conductivity measurements using a Lab-Line Bionmeter. Disc gel electrophoresis was conducted in a Bio-Rad Model 300A cell driven by a model 400 power supply. Gel scanning was performed on a Gilford 2410-S Linear Transport. Enzymatic activity in crude fractions was determined by the radioactivity assay method of Ozanne et al (7). Purified fractions were assayed spectrophotometrically at 340 nm and 25°C using a Gilford model 240 spectrophotometer and a Leeds and Northrup XL-610 recorder, by linking ADP production to pyruvate kinase and lactate dehydrogenase. Reaction mixtures contained 1.9 units pyruvate kinase, 1.6 units lactate dehydrogenase, 0.1 μmole DPNH, 0.4 μmoles phosphoenol pyruvate, 5 μmoles magnesium acetate, 10 μmoles potassium acetate, 0.2 μmoles ATP, 0.066 μmoles kanamycin, 25 μmoles tris-HCl pH 7.8, and 0-0.1 units of neomycin phosphotransferase in a total volume of 0.5 ml. Control assays were conducted in the absence of kanamycin. Units of enzyme were defined as μmoles product formed per minute. Assay chemicals were obtained from Sigma.

RESULTS AND DISCUSSION

The yield of neomycin phosphotransferase obtained by osmotic shock varies considerably as a function of the growth curve of

[3] E. coli JR66/W677 (2-4) has the same parental origin as E. coli JR76,2/W677 (J. Davies, personal communication).

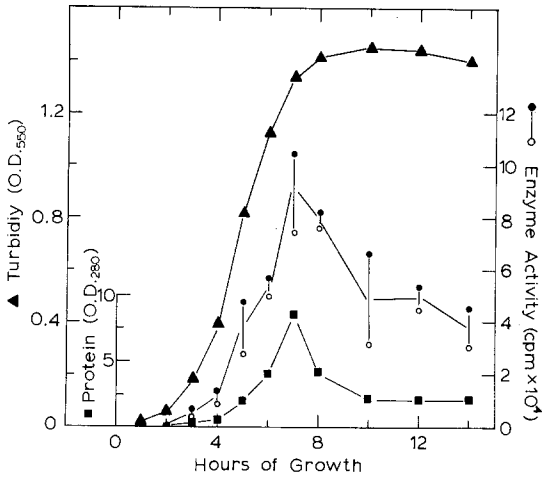

FIGURE 1: Release of neomycin phosphotransferase and protein by osmatic shock as a function of bacterial growth. After addition of 1% overnight inoculum, protein and enzymatic activity were determined on osmatic shockates prepared from 40 ml culture samples, harvested at the times indicated. The open and closed circles represent neomycin phosphotransferase activity obtained in the presence and absence of 0.5 mM magnesium, respectively.

E. coli, reaching a peak between log and stationary phase, then dropping to half the peak level in stationary phase (Figure 1). These results are similar to gentamicin adenylyl transferase (5) which peaks in early stationary phase but drops to a quarter of peak level, and similar to gentamicin acetyl transferase (6) which peaks in late log phase but drops to undectable levels. Differences between the activity profiles of the three enzymes suggest small differences in their periplasmic location. In contrast, Nossal and Heppel (8) observed no decrease in the amount of periplasmic enzymes catalyzing hydrolytic reactions, released by osmotic shock from E. coli in stationary versus logarithmic phase, except under conditions incorporating magnesium in the shock medium. As shown in Figure 1, magnesium had only a small effect on the release of the phosphorylating enzyme, occuring throughout the growth curve. The magnesium-dependent differences between activity profiles of hydrolytic and aminoglycoside inactivating enzymes suggest large differences in their periplasmic location.

The first chromatographic step in the purification of neomycin phosphotransferase employs DEAE agarose. This is also the key

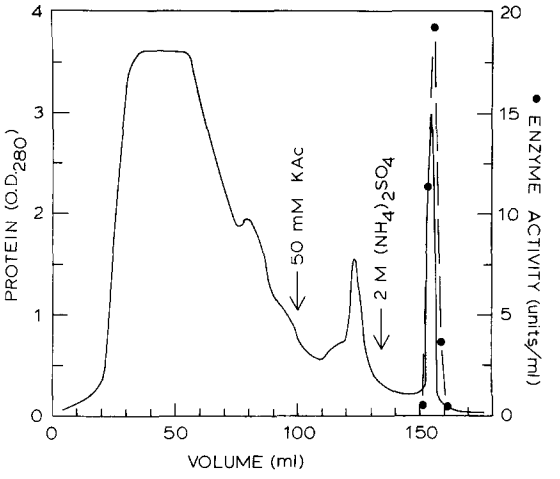

FIGURE 2: Affinity chromatography of neomycin phosphotransferase II on gentamicin C_1a-Affi-Gel 10. 250 mg of protein obtained from a first DEAE agarose column were added to a 1 x 30 cm column and eluted at a flow rate of 0.5 ml/min.

step in the purification of gentamicin adenylyl transferase (5), and serves to separate the two enzymes in extracts from bacteria containing R-factors which direct the synthesis of both. Separation is necessary at this stage since it has not yet been possible to elute the adenylylation enzyme from gentamicin affinity columns. However, the step is neither necessary nor significantly advantageous in the purification of the phosphorylating enzyme.

The key step in the purification of neomycin phosphotransferase is affinity chromotography on gentamicin C_1a-Affi Gel 10. A 20-fold purification is achieved by this step, estimated from the protein profile of Figure 2. Specific activities of peak fractions varied from 2-13 units/mg in four separate preparations. These activities are considerably higher than those obtained by Matsuhashi *et al* (4) from affinity columns. The difference is probably dependent upon the inclusion of a spacer between the antibiotic and agarose during preparation of affinity resins. Umezawa and his associates (4,9) and others (10) have shown that in the absence of a spacer, aminoglycoside inactivating enzymes elute from antibiotic affinity columns within salt gradients (which is atypical of affinity chromotography (11)) of moderate ionic strength in similar fashion to the elution of

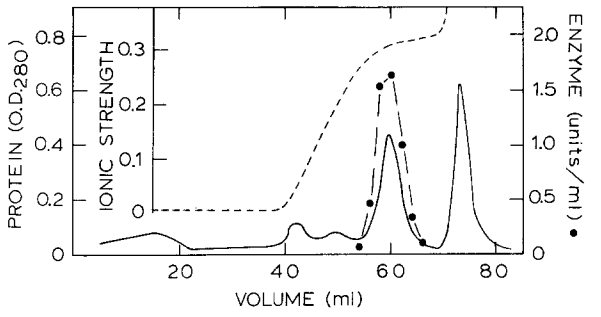

FIGURE 3: Ion-exchange chromotography of neomycin phosphotransferase II on DEAE agarose. 5.6 mg of protein obtained from the affinity column were added to a 1.5 x 30 cm column and eluted at a flow rate of 0.5 ml/min. The salt gradient was generated with equal volumes (27 ml) of standard buffer and standard buffer plus 0.3 M potassium acetate. The final protein peak was eluted with 0.3 M ammonium sulfate.

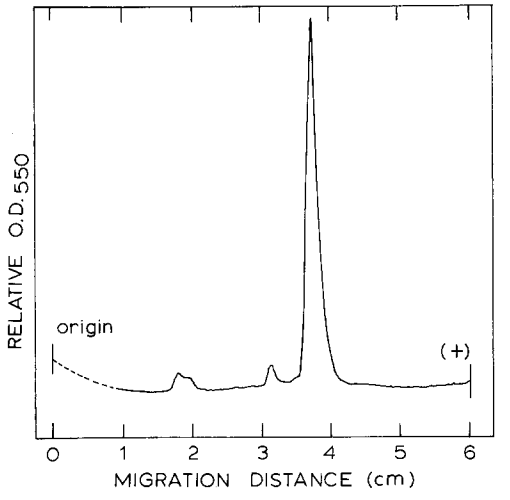

FIGURE 4: Disc gel electrophoresis scan of purified neomycin phosphotransferase II. Approximately 50 μg of the peak fraction from DEAE agarose chromotography were placed on 7.5% polyacrylamide gels and run at pH 8.9 and 3 milliamps/tube. Gels were stained for protein in a solution containing 0.1 g coomassie blue, 45 ml methonol, 45 ml H_2O and diluted 3-fold with water just before use. Destaining was carried out in 10% TCA for 12 hours.

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these enzymes from DEAE ion-exchange resins (3,5,6). In contrast, we have found that inclusion of a spacer renders elution by high ionic strength extremely difficult. Once bound to a gentamicin C_1a-Affi-Gel 10 column, gentamicin adenylyl transferase cannot be eluted by high concentrations of salt, gentamicin acetyl trasfer-

ase elutes as a very broad dilute peak (6), and neomycin phosphotransferase elutes in a full column volume using a concentration of ammonium sulfate which approaches the limits of sulubility of the protein (Figure 2). It therefore appears that a spacer is necessary to accomplish the firm and specific binding of aminoglycoside enzymes, normally attributed to affinity chromotography.

The final step in the purification is ion-exchange chromotography on a second DEAE-agarose column, shown in Figure 3. Gel electrophoresis of active fractions produces three protein bands, two minor components and a major band comprising 90% of the added protein, as shown in Figure 4. Assays of gel slices from parallel gels show all the phosphotransferase activity migrating with the major band. A 3-fold purification is achieved by the final step, estimated from the protein profile of Figure 3.

Specific activities of peak fractions were nearly constant within a single preparation, but varied from 3-10 units/mg between different preparations. In only one preparation was the specific activity increased by the final purification step reaching a value of 10 units/mg, and this occurred with the preparation having the lowest specific activity after affinity chromotography. It appears that contaminant proteins exert a stabilizing effect on neomycin phosphotransferase II. Once the enzyme has been eluted from the affinity column, it becomes highly unstable, losing as much as 50% of its activity in less than a week. Attempts to stabliize the enzyme have not been successful.

Calculations of intermediate specific activities, recoveries, and overall fold of purification were complicated by the instability of purified fractions and also by the inaccuracy of the radioactivity assay. However, the overall purification can be estimated at 300-400-fold on the basis of protein fractionation. In our hands the radioactivity assay lacks linearity with time and enzyme concentration. Similar results were obtained with the radioactivity assay for adenylylation (5). Nevertheless, the radioactivity assay is necessary for semi-quantitative detection of the enzyme in early stages of purification. Substantial ATPase activity dominates the spectrophotometric assay in these initial fractions, but passes through gentamicin affinity

columns. Following this step, background rates in control assays were small (~0.002 ΔOD/min) and independent of the protein concentration in assayed fractions. Spectrophotometric assays of neomycin phosphotransferase II activity following affinity chromotography were linear with time and enzyme concentrations. Use of this assay should greatly facilitate the characterization of this and other aminoglycoside antibiotic phorphorylating enzymes.

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