PARTIAL PURIFICATION AND CHARACTERIZATION OF A BACTERIAL ENZYME CATALYZING REDUCTIVE CLEAVAGE OF ANTHRACYCLINE GLYCOSIDES

Michael McCarville
Dept. of Chemistry
Western Michigan University
Kalamazoo, Michigan 49007

Vincent Marshall
The Upjohn Company
Kalamazoo, Michigan 49001

Received October 16, 1976

SUMMARY: A bacterial enzyme catalyzing the NADH-dependent reductive cleavage of certain anthracycline glycosides has been partially purified. The enzyme is acidic, stable in solution and has an estimated molecular weight of 35,000. The enzyme activity is strongly inhibited by molecular oxygen but not by cyanide or EDTA. No evidence has been found for an enzyme system or associated elements of electron transport.

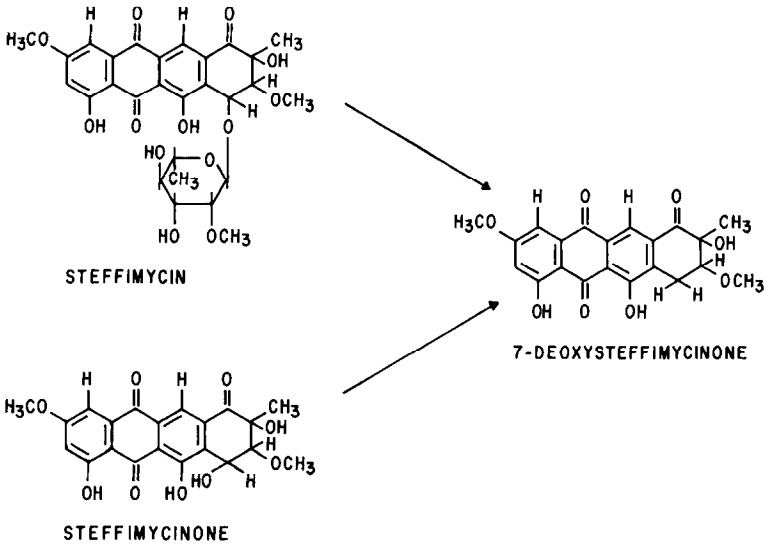
INTRODUCTION

An NADPH-dependent pathway producing 7-deoxyaglycones from anthracycline glycosides has been reported in mammalian systems (1). A cell-free extract from Aeromonas hydrophila catalyzing the same type reaction but preferring NADH has been studied in this laboratory (2)*. No anthracycline aglycone or other intermediates were observed although steffimycinone was found to serve as a substrate. In view of the potentially novel character of the reaction, work was begun to determine whether an enzyme system (e.g. a hydrolase, lyase, reductase combination) or a single cleaving reductase is involved.

METHODS AND MATERIALS

Unless otherwise indicated, "buffer" will refer to a pH 7.4 potassium phosphate buffer solution containing 10 mM 2-mercaptoethanol and 0.02% NaN_3. Purification of the Enzyme. 50 g (wet wt.) of frozen A. hydrophila cells were suspended in 100 ml of 100 mM buffer, sonicated 10 minutes and centrifuged 2 hours at 27,000 x g to obtain 122 ml of a clear, straw-colored supernatant. All purification steps were performed between 1-4°C. The cell-

* (Reaction 1)



free extract was then stirred into 244 ml of DEAE-cellulose slurry equilibrated with 50 mM buffer. After standing 30 min. with occasional stirring, the mixture was washed three times with 244 ml of 50 mM buffer and the washings discarded. Two washings with 244 ml of 250 mM buffer were combined and an ammonium sulfate fractionation was made between 25 and 50% saturation. After centrifugation, the pellet was dissolved in a minimal amount of 50 mM buffer. 8.0 ml of this solution was applied to a 2.5 x 81 cm Ultrogel 44 column for desalting and molecular sieving. 50 mM buffer was used for elution and 4 ml fractions were collected. The most active fractions were combined, applied to a 1 x 11 cm DEAE-cellulose column equilibrated with 50 mM buffer and eluted with 50 ml of a linear gradient between 50 mM and 400 mM buffer. 2 ml fractions were collected, the most active fractions were dialyzed against 50 mM buffer.

Assay. Each assay tube contained 0.1 ml enzyme sample, 0.1 ml 1 M buffer, 0.2 ml 20 mg/ml NADH in 50 mM buffer, 0.55 ml 10 mM 2-mercaptethanol and 0.05 ml 25 mg/ml steffimycin in dimethyl formamide (added last). Assay tubes were placed in a vacuum desicator containing a small quantity of water to prevent evaporation of the assay mixture. After two cycles of evacuation and filling with argon, the desicator was filled with argon at atmospheric pressure and placed in a 37°C incubator for 3 hours. Following incubation, each tube was extracted with 1 ml chloroform. TLC on silical gel using 95:5 chloroform:methanol was used to identify active samples. Quantitative analysis of 7-deoxysteffimycinone product was accomplished by high pressure liquid chromatography, detection was by absorbance at 254 nm (2). Enzyme activity is based on this absorbance. Concentration of enzyme was determined by the Lowry method (3) using bovine serum albumin as standard.

Electrophoresis. Enzyme purification steps were followed using Bio-Rad precast 4% polyacrylamide gels with basic pH 8.9 Bio-Phore buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) employed Bio-Rad 7.5% gels*. Preparative electrophoresis was run on 1.5 mm x 10 cm x 14 cm 5% acrylamide slabs. Slabs were cut into 9 mm wide strips, broken up in a tissue press and eluted into 50 mM buffer. These solutions were then concentrated on Amicon B15 membrane units before assay.

*Coomassie Blue was used to stain the protein.

TABLE 1. Stepwise Purification of the Enzyme

	Volume (ml)	Total Protein (mg)	Total Activity (nanomoles/min)	Yield (%)	Specific Activity (nmoles/min-mg)	Purification (n-fold)
Cell-free Extract	104	2309	1740	100	0.754	---
DEAE-cellulose (batch)	435	653	774	44	1.19	1.6
$(NH_4)_2$ SO_4 concentration, Molecular Sieve Fractionation	28	23	328	19	14.3	19
DEAE-cellulose (gradient)	2	0.4	29.2	1.7	73	97

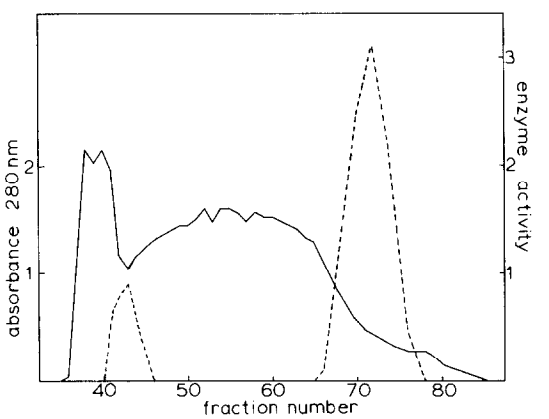


FIGURE 1. Fractionation by Ultrogel 44. Absorbance at 280 nm (——) and enzyme activity (----).

Molecular Weight Determination. Estimates of molecular weight were made by calibration of the molecular sieve column and SDS-PAGE with aldolase, bovine serum albumin and chymotrypsinogen A.

RESULTS and DISCUSSION

Stepwise purification is shown in Table 1. Yields have not been optimized but represent 20% recovery after molecular sieving and 2% at the highest purity.

Fig. 1 indicates that some activity is associated with high molecular weight (>2 x 10^5 daltons) species. Both molecular sieve and SDS-PAGE

determinations place the molecular weight of the activity eluting later at approximately 35,000 daltons. Rechromatography of the high m.w. activity on a 1.3 x 55 cm Ultrogel 44 column after overnight equilibration in buffer indicated no tendency for the activity to dissociate from the large particle.

The color of the precipitated enzyme is white and the visible and UV spectra give no indication that flavins, iron-sulfur proteins or hemes are present. Combinations of various fractions during purification with other fractions or with cell-free extract did not result in activity above that expected for the sums of the components.

Electrophoresis of the purified enzyme by PAGE resulted in four bands of dye absorption. Preparative slab gels were used to identify the active band as the protein which had migrated the greatest distance (nearest the anode) and which absorbed the most dye. Density tracing of this gell suggests the enzyme is approximately 50% pure.

The enzyme is quite stable over a period of 2 weeks in 50 mM buffer. However, the reaction is very sensitive to molecular oxygen. Cell-free extracts show moderate activity when aerobically incubated, presumably because of anaerobic conditions in part of the assay mixture, but more purified preparations show activity only anaerobically. The assay used yields a linear enzyme concentration vs. velocity plot and maintains initial velocity conditions from 1-5 hours after beginning incubation. However, product concentration vs. time extrapolates to 0.5 hours, indicating a lag before initial velocity conditions prevail. During attempts to develop a spectrophotometric NADH assay, it was noted that NADH was consumed very rapidly for several minutes by a reaction requiring steffimycin but yielding no colored products by TLC. This reaction and/or residual oxygen may account for the initial lag. However, preincubation of assay mixtures in individual experiments, each without one of the components of the assay, was not effective in eliminating the lag period. In like manner, addition of sodium dithionite to eliminate residual oxygen had no effect on the lag

period. This difficulty, coupled with low solubilities of substrate and product and the overlapping spectra of the anthracycline and coenzyme chromophores, has precluded the gathering of meaningful kinetic data.

Assays involving 10^{-3} M CN^- and 10^{-2} M EDTA revealed no significant effects on the reaction velocity. Partial inhibition was observed with 10^{-3} M iodoacetamide and 3×10^{-5} M p-chloromercuibenzoate.

Activity of the enzyme begins at approximately pH=5 and rises to a broad optimum in the range pH=7.2-9.5.

REFERENCES

1. Bachur, N. R., and Gee, M. (1976) J. Pharmacology & Experimental Therapeutics, 197, 681-686
2. Marshall, V. P., Reisender, E. A., Reineke, L. M., Johnson, J. H. and Wiley, P. F. (1976) Biochemistry , 15, 4139-4145
3. Lowry, O. H., Rosbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275