JOURNAL OF BACTERIOLOGY, Mar. 1969, p. 1160-1164
Vol. 97, No. 3

Separation of Folic Acid Reductase from *Streptococcus faecium* (ATCC 8043)[1]

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Received for publication 18 November 1968

A strain of *Streptococcus faecium* (ATCC 8043) which is highly resistant to the antifolic acid compound, amethopterin, was gently ruptured by exposing protoplasts of the organism to a hypotonic solution. The crude lysate resulting therefrom was treated by various chemical and physical techniques designed to separate folic acid reductase from dihydrofolic acid reductase. In the process, the enzyme was purified approximately 160-fold; however, throughout the process, the enzyme preparation maintained the ability to reduce folic acid to tetrahydrofolic acid. Attempts to isolate mutants showing a deficiency in either folic acid reductase or dihydrofolic acid reductase were unsuccessful. Based on these results, it is concluded that folic acid is reduced to tetrahydrofolic acid by one enzyme in *S. faecium* (ATCC 8043). The crude lysate was also subjected to ultracentrifugation. An analysis of the supernatant fluid and the sediment indicated that the reductive activity is located in the soluble fraction of the cell.

In 1961 Blakley and McDougall (3) reported the isolation and purification from *Streptococcus faecalis* of an enzyme that specifically catalyzed the reduction of dihydrofolic acid (DHFA) to tetrahydrofolic acid (THFA). They did not observe any reduction of folic acid (FA). More recently, Albrecht, Palmer, and Hutchison (1) showed that partially purified enzyme preparations from two strains of *S. faecalis* had slight activity for reducing FA to THFA. After reinvestigating their system by using a strain of *S. faecalis* that was resistant to antifolic drugs, Hillcoat and Blakley (7) were able to show results similar to those of Albrecht et al. (1).

Since the present report was completed prior to the publication of Hillcoat and Blakley (7), the impetus for this investigation was based on the earlier findings of Blakley and McDougall (3). It seemed logical that *S. faecalis* must have some means for reducing FA, which is a growth factor for *S. faecalis* and must be reduced to be biologically active.

The present report presents data to support the finding that FA is reduced to THFA in *S. faecalis*. Furthermore, the evidence suggests that the reduction is catalyzed by a single enzyme.

[1] Work reported is part of a dissertation submitted by E. L. Speck to the faculty of the Graduate Council, The George Washington University, in partial fulfillment of the requirements for the Ph.D. degree.

MATERIALS AND METHODS

Organism. The organism used in this study was *S. faecalis* ATCC 8043. According to the criteria set forth by Deibel, Lake, and Niven (4), this organism should be classified as *S. faecium*. The strain of *S. faecium* (Sf/AM) used in the present study is about 1,000 times more resistant to amethopterin than is the parent strain (14).

Growth medium. *S. faecium* was prepared for harvesting by growth in 20 to 30 liters of Difco Folic Acid Assay Medium containing (final concentration) 2 ng of folic acid per ml.

Preparation of cell-free extract. R. C. Wood (*personal communication*) found that he could prepare protoplasts from Sf/AM. This was accomplished in the following manner: 20 to 30 liters of Folic Acid Assay Medium were inoculated with the organism and incubated at 37 C until the turbidity, measured in a Klett-Summerson photoelectric colorimeter at 660 nm (filter no. 66), reached 65 Klett units. This reading was equivalent to 2.4×10^8 viable units/ml. Upon reaching the desired turbidity, the cells were harvested in a Sorvall RC-1 continuous-flow centrifuge. The cells were then washed three times in 50- to 60-ml portions of a 1.0 M solution of sucrose (containing 0.002 M $MgCl_2$). After the third washing, the pellet was suspended in 100 to 130 ml of the same hypertonic solution and placed in a water bath (37 C). Turbidity readings were taken every hour for 3 to 4 hr. This was accomplished by diluting 0.1 ml of the cell suspension in 5.0 ml of 0.01 M Sorensen's phosphate buffer (*p*H 6.5). If protoplasts formed, there was a substantial

drop in turbidity in 2 to 3 hr, becoming stable in 3 to 4 hr.

The protoplasts were lysed by suspension in 50 to 60 ml of "lysing solution" containing 0.003 M $MgCl_2$ in 0.01 M Sorensen's phosphate buffer (*p*H 7.0). The suspension was allowed to stand at room temperature for 15 to 20 min with occasional gentle shaking in a Vortex Junior mixer. The cell debris was separated from the lysate by centrifugation at 17,000 × *g* for 15 min. All subsequent steps in the separation procedure were done either in an ice bath or a cold room.

Chemical determinations. Protein, nucleic acids, and carbohydrate were determined by the methods described by Seibert and Affronti (12).

Nucleic acid precipitation. Nucleic acids were precipitated by the use of 25% $MnCl_2$. This was added dropwise to the crude lysate while stirring. After the precipitate was eliminated by centrifugation, the lysate was dialyzed against 7 volumes of distilled water containing 1 mM ethylenediaminetetraacetic acid (Fisher Scientific Co.) for 9 to 10 hr. The material was partially reconcentrated by placing the dialysis bag, containing the lysate, in Carbowax (ethylene glycol 20,000; Fisher Scientific Co.) for 5 to 6 hr.

Protein precipitation. Extraneous protein was eliminated by adjusting the *p*H of the lysate to 4.0 to 4.3 with 0.05 M acetate buffer (*p*H 4.0). After centrifugation, the supernatant fluid was treated for 30 min with 1 to 2 g of Norit A (activated charcoal) to remove pteridine materials by adsorption. This was then filtered by using fine filter paper.

Ion-exchange chromatography. Diethylaminoethyl (DEAE) cellulose resin was charged with 0.01 M Sorensen's phosphate buffer (*p*H 6.5). The resin was washed with 1-liter portions of distilled water until phosphate could not be detected by the method of Fiske and SubbaRow (5). The charged resin was poured into a glass column (3 by 40 cm) to a height of 18 cm. The partially purified extract was placed on the column and allowed to percolate into it. The following Sorensen's phosphate buffers, each at *p*H 6.5, were applied in 100-ml quantities: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, and 0.25 M. The flow rate was adjusted to 2 ml/min by positive pressure with argon gas, and 5-ml fractions were collected.

Ultraviolet irradiation. Agar plates of Folic Acid Assay Medium containing 10 ng of citrovorum factor (CF) per ml were swabbed with a washed suspension of strain Sf/AM. The plates were irradiated for 40 sec with a 15-w desk lamp at a distance of 20 cm. The plates were incubated at 37 C for 48 to 72 hr. The 30 to 40 colonies which appeared on the agar surface were replicated (9) to agar plates containing 2 μg of FA/ml. Mutants were identified as colonies capable of utilizing CF and incapable of reducing FA to THFA.

Mutagenic treatment. An overnight culture of strain Sf/AM (approximately 10^7 viable particles) was washed and resuspended in 5.0 ml of 0.1 M Sorensen's phosphate buffer (*p*H 7.0) to which 0.3 ml of ethyl methane sulfonate (EMS) was added. The suspension was held at room temperature for 20 min. The suspension was then diluted with an additional 5.0 ml of buffer and immediately centrifuged. The cells were washed three times with 10-ml quantities of buffer. Finally, the cells were suspended in 5.0 ml of Folic Acid Assay Medium containing 10 ng of FA/ml. The suspension was placed in a water bath (37 C) for about 1 hr; penicillin was then added to a concentration of 300 units/ml. The system was incubated at 37 C for 10 hr. At the conclusion of this incubation, penicillinase was added to a concentration of 1 mg/300 units of penicillin. The penicillinase was allowed to react for 2 hr at 37 C. Samples were removed and streaked onto agar plates of Folic Acid Assay Medium containing 10 ng of CF/ml. Colonies were evident after 24 to 48 hr at 37 C. These colonies were replicated (9) to agar plates containing Folic Acid Assay Medium and 2 ng of FA/ml and incubated at 37 C for 24 hr.

EMS was chosen as a mutagenic agent because of the high level of survival (10).

Ultracentrifugation. Particulate and soluble aspects of the crude lysate were separated by means of an ultracentrifuge. The crude lysate was prepared in the usual manner and then centrifuged at 144,000 × *g* for 2 hr in a Spinco (model L) ultracentrifuge.

Reaction system for conversion of FA to THFA. The reaction system consisted of 0.7 ml of the enzyme preparation. The final protein concentration varied from 40 to 50 μg/ml to 4 to 5 mg/ml, depending on the step in the extraction procedure at which the extract was assayed. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was added to a final concentration of 0.3 mM. The final concentration of sodium ascorbate was 0.03 M. The final concentration of folic acid was 200 ng/ml. The total volume of the reaction system was 1.0 ml. The system was incubated in a water bath (37 C) for 1 hr, and the material was immediately assayed. Appropriate controls clearly showed that the reduction of FA is enzyme-dependent and does not occur nonenzymatically with the concentrations of NADPH and ascorbate used in these studies.

One unit of enzyme is defined as the amount (micromoles) of THEA formed per minute at 37 C. Specific activity is defined as units of enzyme per gram of protein.

Assay method. THFA was assayed by the procedure of Bakerman (2). Briefly, this procedure consisted of sterilizing 2.5 ml of double-strength CF assay medium in tubes (15 by 10 mm). After cooling, 1.0 ml of sodium ascorbate was added to a final concentration of 0.03 M. Samples, usually 0.02 or 0.04 ml, were taken from the reaction system and added to the assay medium. These were subsequently inoculated with 0.1 ml of a 1:100 dilution of washed cells of *Pediococcus cerevisiae* ATCC 8081. The tubes were than brought to 5.0 ml with sterile distilled water. The assay was incubated at 37 C for 18 to 20 hr. Growth response was measured as turbidity in a Klett-Summerson colorimeter (filter no. 66).

RESULTS

Enzyme extraction by chemical means to separate reductase activity. During the extraction procedure, the enzyme preparations were assayed

microbiologically and protein determinations were carried out (Table 1).

During the purification procedure, there was a substantial increase in the number of recoverable units of enzyme. At the end of charcoal treatment, for example, there was a 23% recovery of enzyme. These results could reflect the elimination of an inhibitor or the elimination of stored pteridines which are capable of stimulating the growth of *P. cerevisiae*. The latter material is presumably eliminated by its adsorption to charcoal, whereas the former is precipitated by acid.

As can be seen from Table 1, the overall effect of these chemical treatments was to increase the specific activity 85-fold by eliminating much of the extraneous protein, acid-precipitable inhibitor, and preformed biologically active pteridine compounds, without separating FA reductase activity from DHFA reductase activity.

TABLE 1. *Results of enzyme extraction procedures*

Prepn	Units of enzyme[a]	Protein concn	Specific activity[b]	Fold increase	Enzyme recovery
		mg/ml			%
Crude lysate..	0.16	4.0	40		
Nucleic acid ppt. after dialysis.....	0.068	2.1	34	−0.15	42
Acid ppt.....	0.27	0.112	2,400	60	168
Charcoal.....	0.38	0.112	3,400	85	237

[a] One unit of enzyme is defined as the micromoles of THFA formed per minute at 37 C.

[b] Units of enzyme per gram of protein.

DEAE cellulose column chromatography. Chromatography revealed six or seven well-separated fractions (Fig. 1). Fractions 63 to 88 apparently contained the majority of the remaining nucleic acid and were clearly separated from other fractions. All fractions were assayed, but activity for the reduction of FA to THFA could be detected only in tubes 54, 55, 56, and, to a small degree, 57 (Table 2). When the fractions were boiled for 10 min, reductase activity was eliminated.

Fraction 55 was eluted with 0.04 M Sorensen's phosphate buffer (*p*H 6.5). This buffer was added after tube 40 was collected and was used to collect fractions 41 to 60. Fraction 55 showed a recovery of about 76% of the enzyme, indicating there had been a sharp separation of the enzyme. Inasmuch as the major portion of the reductive enzyme appeared in a narrow region of the elution pattern, probably only one enzyme was involved.

Fraction 55 represented an increase in specific activity over the crude lysate of about 160-fold. Although the degree of purity was not absolutely established, chemical analyses of these fractions

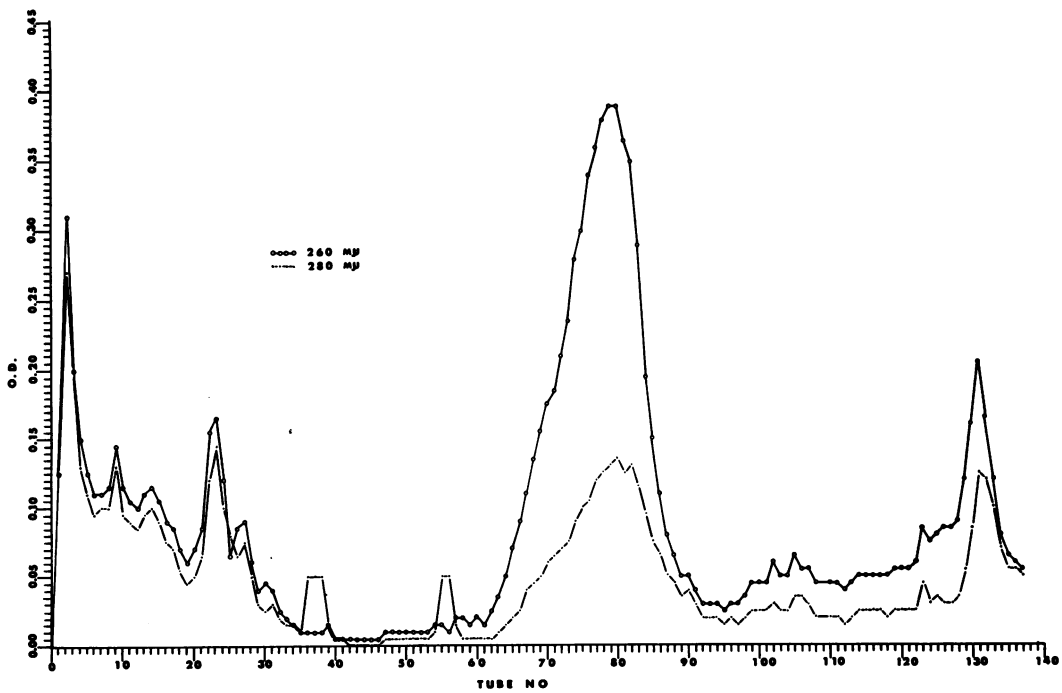

FIG. 1. *Spectrophotometric pattern of eluates from DEAE cellulose.*

indicated (Table 3) that fractions 54 to 57 were contaminated with carbohydrate and, to a lesser extent, deoxyribonucleic acid (DNA).

Isolation of mutants. It was reasoned that if an FA reductase and a DHFA reductase did exist, they would be controlled by separate genes. Theoretically, then, it would be possible to recover mutants lacking one or the other enzyme. After the examination of about 10,000 colonies, mutants requiring CF for growth because they either lack FA reductase or DHFA reductase could not be demonstrated.

Ultracentrifugation studies. It has been shown that microorganisms capable of taking up FA are also capable of reducing it (13); conversely, those microorganisms which do not assimilate FA have only a limited capacity to reduce FA. This evidence suggested the possibility that the reductive enzyme(s) also acts as a permease. To examine this possibility, ultracentrifugation was employed to separate particulate matter from the soluble complex of the crude lysate. Both fractions were assayed microbiologically; 80 to 85% of the enzyme units were recovered in the soluble fraction (Table 4). Activity could not be detected in the sediment. Thus, it appears that the enzymatic activity for reducing FA to THFA resides in the soluble fraction of the cell.

DISCUSSION

Conflicting results have been reported regarding the enzymatic components reducing FA to THFA.

TABLE 2. *Assay of fractions eluted from DEAE cellulose*

Fraction	Units of enzyme	Protein concn	Specific activity	Fold increase over crude lysate	Enzyme recovery
		mg/ml			%
54	0.003	0.063	49	1.2	2
55	0.29	0.043	6,300	160	76
56	0.006	0.056	110	3	2
57	0.00022	0.05	4.4	—[a]	0.1

[a] Decrease.

TABLE 3. *Chemical analysis of fractions eluted from DEAE cellulose*

Fraction	Protein	DNA	Carbohydrate
	mg/ml	*mg/ml*	*mg/ml*
54	0.063	0.0041	0.036
55	0.043	0.0013	QNS[a]
56	0.056	0.0055	0.018
57	0.05	0.0051	0.012

[a] Quantity not sufficient.

TABLE 4. *Enzymatic activity of crude lysate before and after ultracentrifugation*[a]

Prepn	Units of enzyme	Protein concn	Specific activity	Enzyme recovery
		mg/ml		%
Crude lysate 1....	0.140	3.8	37	(100)
Ultracentrifuged lysate 1.....	0.120	2.3	52	85
Crude lysate 2....	0.170	8.8	19	(100)
Ultracentrifuged lysate 2.....	0.140	4.2	33	82

[a] Crude lysate was subjected to ultracentrifugation at 144,000 × *g* for 2 hr.

Some of the earlier reports on chicken liver FA reductase indicated that FA was reduced to THFA by two separate enzymes (6, 11). On the other hand, S. F. Zakrzewski (*unpublished data*) reported that his preparation from chicken liver was capable of reducing both FA and DHFA, although the enzyme appeared to be more specific for the latter compound. These results have been verified by Kaufman and Gardiner (8).

A similar situation exists with the bacterial FA reductase system. Blakley and McDougall (3) could detect only DHFA reductase activity when they assayed their enzyme prepared from *S. faecium* 8043. (This strain was sensitive to amethopterin.) Albrecht et al. (1) prepared, from a strain of *S. faecium* that was resistant to amethopterin, an enzyme extract which was capable of reducing FA to DHFA. A similar enzyme preparation from a sensitive strain was considerably less active. More recently, Hillcoat and Blakley (7) showed that an enzyme preparation from a resistant strain of *S. faecalis* was capable of reducing FA to THFA. Their evidence strongly suggests that two enzymes are involved, with different affinities for FA and DHFA, as was found in chicken liver (Zakrzewski, *unpublished data*).

The evidence presented in this communication supports the view that a streptococcus is enzymatically equipped to reduce FA to THFA. However, the chemical and physical procedures utilized in this investigation, which are capable of separating proteins with small difference in isoelectric points and charges, suggest that only one enzyme is involved. DEAE cellulose, for example, is known to separate proteins of slight charge differences. In spite of this sensitive technique, the reductase activity could not be separated. Furthermore, the results of the DEAE column chromatography showed that the major portion of the reductase can be eluted rather sharply (Fig. 1). It seems reasonable to conclude that, if two enzymes do indeed exist in this system, they must have the same

charge and similar sizes and shapes, which is unlikely. In addition, the results of mutant isolation also suggest that only one enzyme is involved.

This evidence does not preclude, however, the existence of two different enzyme systems. That is to say, one system may consist of a single enzyme limited in its capacity to reduce DHFA to THFA. A second system may consist of one enzyme capable of reducing both FA and DHFA. This latter system probably is in much greater concentration in the resistant organism than in the sensitive organism. This concept, together with the fact that various methods of purification and separation have been used, may account for the apparent contradictions of results by various investigators.

During the chemical steps in the purification of the crude lysate, the enzyme units increased markedly after acid precipitation (Table 1). It appears that more enzyme is recovered than was contained in the starting material. It is possible that acid precipitation eliminates, among other things, a protein inhibitor. The inhibitor could have masked the effect of the enzyme present in the crude lysate, so that there was an apparent increase in enzyme units with the elimination of the inhibitor. There was a further, although smaller, increase in enzyme units after charcoal treatment. This was probably due to the removal of stored preformed pteridines, which are biologically active and preferentially utilized for growth. This certainly would be reflected in the biological assay.

It appears that the total enzyme activity is located in the soluble cell fraction. On this basis it is difficult to imagine that this enzyme may also be a permease as some have suggested (13).

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