Purification of Thymidine-diphospho-glucose 4,6-Dehydratase from an Erythromycin-producing Strain of *Saccharopolyspora erythraea* by High Resolution Liquid Chromatography*

(Received for publication, March 25, 1988)

Jesus A. Vara and C. Richard Hutchinson

From the School of Pharmacy and the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

TDP-D-glucose 4,6-dehydratase was purified from *Saccharopolyspora erythraea*, the producer of the macrolide antibiotic erythromycin, by a high resolution chromatographic method that exploited the difference in the behavior of the enzyme on anionic exchange chromatography in Tris/HCl or phosphate buffers. By this method, the enzyme was purified approximately 900-fold by two anionic exchange steps to more than 90% homogeneity. It was further purified to apparent homogeneity by hydrophobic interaction chromatography. The enzyme is a homodimer of M, 36,000 subunits, is highly specific for TDP-D-glucose, requires NAD⁺ as cofactor, and shows a Kₘ of 34 μM and Vₘₐₓ of 26 μmol h⁻¹ mg⁻¹ of protein for TDP-D-glucose. TDP and TTP strongly inhibit the enzyme at 2 mM. The maximal TDP-D-glucose 4,6-dehydratase activity coincides with the time of erythromycin production, suggesting that this enzyme is involved in antibiotic biosynthesis.

Macrolide antibiotics are made in bacteria largely from simple fatty acids and glucose (1). For erythromycin, the macrolide antibiotic produced by *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus* (2)), it has been established that its two deoxy sugars, D-desosamine and L-mycarose, are made from D-glucose (3, 4). Although the specific steps used in the conversion of glucose to these two deoxy sugars are not known, the biosynthesis of other deoxy sugars like rhamnose and fucose is well-understood and involves nucleotidyl diphosphate esters of glucose (5) or mannose (6). The first step in these pathways is the oxidation of a nucleotidyl diphosphoglucose to its 4-keto-6-deoxy derivative. Enzymes that catalyze this oxidation have been purified from *Escherichia coli* (7), *Pseudomonas aeruginosa* (8), and *Pasteurella pseudotuberculosis* (9) as well as from plant (10) and mammalian (11) sources. This type of enzyme has also been studied in other antibiotic-producing bacteria (12, 13), and one report describes the partial purification of thymidine-diphospho-glucose 4,6-dehydratase from *Streptomyces rimosus*, a producer of the macrolide antibiotic tylosin (13).

Deoxy (amino) sugar formation has a key role in the biosynthesis of the macrolides and several other types of antibiotics (14). To further our investigation of the genetics and biochemistry of erythromycin production, we purified and characterized TDP-D-glucose 4,6-dehydratase from *S. erythraea*. The purification was accomplished with fast protein liquid chromatography by exploiting the different behavior of the enzyme on anionic exchange chromatography in the presence of Tris/HCl and phosphate buffers.

**MATERIALS AND METHODS AND RESULTS**

We observed an exceptional dependence of protein resolution by fast protein liquid chromatography on the eluting buffer during the purification of TDP-D-glucose 4,6-dehydratase from *S. erythraea*. Because of the different behavior of this enzyme on anionic exchange chromatography in Tris/HCl or phosphate buffers, we were able to purify it using only three high resolution chromatographic steps. This buffer effect appears to be general based on the behavior of another protein fraction from *S. erythraea* (Fig. 1), where an essentially single peak could be resolved by rechromatography in many peaks simply by changing to a phosphate buffer. Furthermore, we have purified an L-valine dehydrogenase from *Streptomyces coelicolor* using the same approach. Whereas the exact reason for our observations is unknown, Scopes (18) has noted that phosphate buffers have been used successfully in anionic exchange chromatography.

Some of the properties of TDP-D-glucose 4,6-dehydratase from *S. erythraea* differ significantly from the enzymes isolated from other bacteria. Although it also is a homodimer of M, 36,000 subunits (the other bacterial enzymes are homodimers of M, 38,000–43,000 subunits (5–9)), the enzyme does not contain a tightly bound nucleotide cofactor and thus requires NAD⁺ for activity. In this respect, the *S. erythraea* enzyme is like the enzymes from *P. aeruginosa* (8) and *P. pseudotuberculosis* (9). The cofactor is not required by the enzyme from *S. rimosus*, although its activity was stimulated by addition of NAD⁺ (13). Several other properties of the two enzymes differ from those found in the other bacterial species.

* Portions of this paper (including "Materials and Methods," "Results," Figs 2–5, and Tables I and II) are presented in Miniprint at the end of this paper. The abbreviations used are: FPLC, fast protein liquid chromatography; DTt, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

† To whom reprint requests should be addressed: School of Pharmacy, University of Wisconsin, 425 N. Charter St., Madison, WI 53706.

‡ Portions of this paper (including "Materials and Methods," "Results," Figs 2–5, and Tables I and II) are presented in Miniprint at the end of this paper. The abbreviations used are: FPLC, fast protein liquid chromatography; DTt, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
though this 6-deoxy sugar is not widely distributed among the formation of cell wall constituents like rhamnose, even buffer using a 0-1 M KCl elution gradient. Buffer A is 100 mM Tris/HCl buffer B is buffer A with 2 M KCl. B, the elution profile of the same fraction after rechromatography under the conditions in A but in a phosphate buffer. Buffer A is 100 mM phosphate, pH 7.5; buffer B is buffer A with 2 M KCl.

Streptomyces enzymes are similar (pH optimum, $K_{m}$ inhibition by TDP). The S. erythraea enzyme has a stricter substrate specificity, lower $K_{m}$ for TDP-D-glucose (33 mM), and significantly smaller $V_{max}$ (25 $\mu$mol h$^{-1}$ mg$^{-1}$) than the E. coli enzyme (70 $\mu$M and 420 $\mu$mol h$^{-1}$ mg$^{-1}$, respectively (7)). Comparison of the respective $V_{max}/K_{m}$ values for these two enzymes, 0.012 versus 0.10 mol$^{-1}$ s$^{-1}$ mg$^{-1}$ of protein, reveals that the S. erythraea enzyme is 10 times less efficient than the E. coli enzyme.

One function of the TDP-D-glucose 4,6-dehydratases from the two Streptomyces could be to provide the first intermediate of the biosynthetic pathway to deoxy sugars like mycardose that are present in the macrolide antibiotics produced by each species. In fact, the formation of TDP-L-mycarose from TDP-D-glucose has been demonstrated in a cell-free system from the tylosin-producing strain of S. rimosus (19). Our observation (see "Results") that the level of the S. erythraea TDP-D-glucose 4,6-dehydratase and erythromycin in S. erythraea. Enzyme activity and antibiotic titer were determined as described under "Materials and Methods." A, erythromycin titer and TDP-D-glucose 4,6-dehydratase activity as a function of culture age. For the change in erythromycin titer with culture age, the titer on 1 day was subtracted from the titer on the following day, and the difference was plotted. B, cell density and erythromycin titer as a function of culture age.

enzyme in erythromycin production will have to come from cloning and gene analysis experiments underway in our laboratory.

Acknowledgments—We are very grateful to Stefano Donadio, Ali Shafiee, and Karl Maurer for their advice throughout this work and Juan Imperial and Vinod Shah (Department of Biochemistry, University of Wisconsin, Madison, WI) for their help with the protein purification.

REFERENCES

1. Omura, S., and Tanaka, Y. (1984) in Macrolide Antibiotics (Omura, S., ed) pp. 199–231, Academic Press, New York
2. Labeda, J. (1987) Int. J. Syst. Bacteriol. 37, 19–22
3. Corcoran, J. W. (1981) Antibiotics (Berl.) 4, 132–174
4. Seno, E. T., and Hutchinson, C. R. (1986) in The Bacteria: a Treatise on Structure and Function (Day, L. E., and Queener, S. W., eds) Vol. 4, pp. 231–279, Academic Press, New York
5. Glaser, L., Zarkowsky, H., and Ward, L. (1972) Methods Enzymol. 28, 446–454
6. Chang, S., Duerr, B., and Serif, G. (1988) J. Biol. Chem. 263, 1693–1697
7. Gilbert, J. M., Matsushashi, M., and Strominger, J. L. (1965) J. Biol. Chem. 240, 1305–1308
8. Kornfeld, S., and Glaser, L. (1961) J. Biol. Chem. 236, 1791–1799

\[3 J. A. Vara and C. R. Hutchinson, unpublished results.\]
TDP-d-glucose 4,6-dehydratase from S. erythraea

Materials and Methods

Organic and Crude Conditions - S. erythraea CASMA was attained from Applet laboratory, NORTH CAROLINA. It grew at 30°C in the yeast-M-52 medium (5). Large scale cultures were grown in a 250-L fermentor at 30°C and 200 rpm on a 50-L starter culture for 24 h after inoculation. The culture was centrifuged for 2 h at 4°C, and the supernatant was used as the source of enzyme.

Enzyme purification - All the following operations were carried out at 4°C except for PFC-2000 (Pharmacia AG, Uppsala, Sweden) chromatography, which was carried out at 25°C. The supernatant was equilibrated with TED buffer (3). The column was washed with about 20 mL of buffer A containing 0.2 M KC1 and this fraction was concentrated to 100 mL by ultrafiltration through a membrane with HR5120 (Amicon Corporation, Beverly, MA). The enzyme solution was filtered through a 0.45-mm membrane and applied to a column (Pharmacia, Uppsala, Sweden) equilibrated with TED buffer. The column was washed with about 20 mL of buffer A (TED buffer) and then the following gradient was made from buffer A and B (TED containing 0.3 M KC1) at 4 ml/min: 100% A for 4.80 min, 80% A to 20% A over 35 min period, and 20% A for 30 min. The active fractions appearing at approximately 200-300 mL (A280 < 0.18) were collected.

Identification of the Purification Products - The active fractions (10% of the total) were treated with two consecutive steps of protein A-Sepharose chromatography. The enzyme was centrifuged at 10,000 rpm for 10 min and then the cell-free extract was recovered.

Other Methods - 14C-NAD was obtained from New England Nuclear. All the other chemicals were obtained from Sigma Chemical Company. Radioactive assays were performed using the techniques described previously (5). All the results were expressed as mean values ± standard error.

Results

Purification of TDP-d-glucose 4,6-dehydratase - The enzyme was purified from S. erythraea by a series of chromatography steps (5). The enzyme was centrifuged at 10,000 rpm for 10 min and then the cell-free extract was recovered.

Other Methods - 14C-NAD was obtained from New England Nuclear. All the other chemicals were obtained from Sigma Chemical Company. Radioactive assays were performed using the techniques described previously (5). All the results were expressed as mean values ± standard error.

Results

Purification of TDP-d-glucose 4,6-dehydratase - The enzyme was purified from S. erythraea by a series of chromatography steps (5). The enzyme was centrifuged at 10,000 rpm for 10 min and then the cell-free extract was recovered.

Other Methods - 14C-NAD was obtained from New England Nuclear. All the other chemicals were obtained from Sigma Chemical Company. Radioactive assays were performed using the techniques described previously (5). All the results were expressed as mean values ± standard error.

Results

Purification of TDP-d-glucose 4,6-dehydratase - The enzyme was purified from S. erythraea by a series of chromatography steps (5). The enzyme was centrifuged at 10,000 rpm for 10 min and then the cell-free extract was recovered.

Other Methods - 14C-NAD was obtained from New England Nuclear. All the other chemicals were obtained from Sigma Chemical Company. Radioactive assays were performed using the techniques described previously (5). All the results were expressed as mean values ± standard error.
TDP-d-glucose 4,6-Dehydratase from *S. erythraea*

**TABLE I**

| Purification of *S. erythraea* TDP-4,6-Dehydratase |  |
|-----------------------------------------------|---|
| Purification | Volume | Total | Specific | Yield | Purification |
| step | activity | activity | activity | | |
| ml | mg | units | % | % | % |
| 5% | 550 | 3630 | 79.9 | 0.022 | (100) | 1 |
| DEAE-cellulose | 700 | 175 | 63.0 | 0.39 | 78 | 14 |
| Mono-Q-Tris | 45 | 17 | 74.2 | 5.1 | 52 | 47 |
| Mono-Q-phosphate | 6 | 1.16 | 31.3 | 20.1 | 40 | 913 |
| Alkylo-Sepharose | 2 | 1.36 | 25.0 | 24.2 | 32 | 1100 |

*Washed, determined as described in "Materials and Methods".*

**TABLE II**

| Inhibitor of TDP-4,6-Dehydratase by Alkaloide§ | Specific activity | % inhibition |
|-----------------------------------------------|------------------|-------------|
| None | 29.2±2.0 | 100 |
| Thymidine | 26.1±2.6 | 84 |
| TDP | 25.9±1.4 | 92 |
| TTP | 2.8±0.5 | 99 |
| TTP | 6.7±0.2 | |

*For each determination, triplicate standard reactions were run with 2.5 μg of purified enzyme and a 2 mM concentration of the inhibitor.*

**FIG. 2.** Alkylo-Sepharose FPLC of the *S. erythraea* TDP-4,6-dehydratase. Conditions were as described in "Materials and Methods." Buffer A is 25 mM sodium acetate, pH 5.0, and buffer B is buffer A with 2 M ammonium sulfate. The main peak, which had the enzyme activity, is shaded.

**FIG. 3.** Silver-stained SDS-PAGE of purification fractions. Lane 1, crude extract (7 μg); lane 2, DEAE-cellulose (5 μg); lane 3, Mono-Q (3 μg); lane 4, Mono-Q-phosphate (2 μg); lane 5, Mono-Q (2 μg); lane 6, Mono-Q-phosphate (2 μg); lane 7, Mono-Q-phosphate (2 μg); lane 8, Mono-Q-phosphate (2 μg). The position of the proteins were stained with the kit from Bio-Rad (Richmond, CA). In lane 1 indicates the position of the native TDP-4,6-dehydratase.

**FIG. 4.** Molecular weight determination of the native TDP-4,6-dehydratase by gel filtration through Sephadex G-15. The column was calibrated in triplicate and the mobility of standard proteins was determined by the arrows at the top, then dialysis followed and developed under the same conditions (0.5 ml/min. flow rate) using PBS buffer. The two peaks were collected independently and analyzed by enzymatic activity and SDS-PAGE as described in Materials and Methods. The major peak exhibited the enzyme activity.

**FIG. 5.** Inhibition of the *S. erythraea* TDP-4,6-dehydratase by TDP and TTP at 2 mM concentrations. Assay conditions are given in "Materials and Methods" and in Table II.