STUDIES ON THE ENZYMATIC SYNTHESIS OF 2,3-DIHYDROXY-N-BENZOYL-L-SERINE IN ESCHERICHIA COLI

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SUMMARY: The enzymatic synthesis of 2,3-dihydroxy-N-benzoyl-L-serine has been resolved into three protein components. Two of these proteins are responsible for the conversion of 2,3-dihydroxybenzoic acid, L-serine, and ATP to cyclo-tris (2,3-dihydroxy-N-benzoyl-L-seryl) which is the cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine. The third protein hydrolyzes this cyclic trimer through intermediate compounds to 2,3-dihydroxy-N-benzoyl-L-serine.

The role of 2,3-dihydroxybenzoic acid[1] and its N-benzoyl-amino acid derivatives as growth factors for various microorganisms has been attributed to their properties of iron chelation (1). The glycine, L-serine, and L-lysine conjugates are excreted by B. subtilis (2), E. coli (3,4), and Azotobacter vinelandii (5), respectively, in response to growth in low-iron media. Recently, Gibson and his coworkers have identified several compounds containing DBA and L-serine in the growth medium of cultures of E. coli grown under iron deficiency (6,7), and they have suggested the interrelationship among these compounds as shown in Fig. 1. These authors (7) have postulated that 2,3-dihydroxy-N-benzoyl-L-serine (DBS) is produced as a result of the hydrolysis of cyclo-tris (2,3-dihydroxy-N-benzoyl-L-seryl) (cyclic DBS $_3$) (compound I) and that this reaction proceeds through compounds II and III, the linear trimer and dimer, respectively, of DBS. Furthermore, it was suggested that only cyclic $(DBS)_3$ was physiologically important. Recently Pollack et al. (8) have also isolated and crystallized cyclic $(DBS)_3$ from the growth medium of Salmonella typhimurium and have shown (9) that it is about 100-fold more effective than DBS in promoting the growth of a mutant which is unable

[1] Abbreviations used: DBA, 2,3-dihydroxybenzoic acid; DBS, 2,3-dihydroxy-N-benzoyl-L-serine; cyclic $(DBS)_3$, cyclo-tris (2,3-dihydroxy-N-benzoyl-L-seryl).

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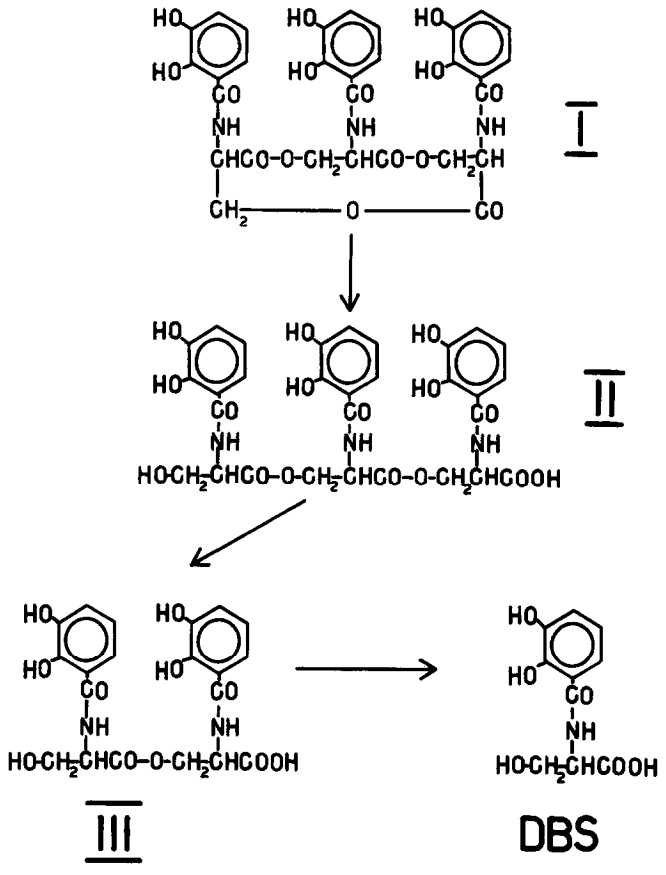

Fig. 1. Proposed metabolism of cyclic $(DBS)_3$. Compounds are: I, cyclo-tris (2,3-dihydroxy-N-benzoyl-L-seryl); II, N, N', N" - tris (2,3-dihydroxybenzoyl)-O-seryl-O-seryl serine; III, N,N'bis (2,3-dihydroxybenzoyl)-O-seryl-serine; DBS, 2,3-dihydroxy-N-benzoyl-L-serine.

to synthesize cyclic $(DBS)_3$.

A previous study (3) has shown that when DBA, L-serine, and ATP were incubated with a cell-free extract from E. coli which had been grown in a low iron medium, the only product of the reaction was DBS. The present report shows that while unpurified cell-free extracts of E. coli converted DBA, L-serine, and ATP to DBS, cyclic $(DBS)_3$ was the major product when a more purified enzyme fraction was employed. An enzyme activity which converts cyclic $(DBS)_3$ to its acidic breakdown products has been detected. In addition, the synthesis of the cyclic trimer requires at least two protein fractions which have been separated on Sephadex G-100.

MATERIALS AND METHODS: E. coli K-12, strain 2276, a methionine-cyano B_{12} auxotroph, was grown in a low iron medium (10), and cell-free extracts were prepared as previously described (4). A 0-40% saturated ammonium sulfate fraction was prepared from the cell-free extract, and after dialysis for 10 hrs against a buffer containing Tris-Cl, 10 mM, pH 7.4, and dithiothreitol, 2.5 mM, was applied to a Sephadex G-100 column.

The incubation mixture used to form DBS and cyclic $(DBS)_3$ contained in a total volume of 0.2 ml: enzyme; Tris-Cl buffer, pH 7.4, 10 μmoles; DBA, 100 nmoles; ^{14}C-L-serine, 100 nmoles (specific activity 1700-1800 cpm per nmole); ATP, 1 μmole and $MgCl_2$, 2 μmoles. Incubations were carried out for 15 min at 37°.

Assay for DBS-Containing Compounds - The reactions were stopped by the addition of 0.8 ml of 0.05 M HCl. All DBS-containing compounds were extracted with 3 ml of ethyl acetate. Two ml of the ethyl acetate layer were removed, added to 10 ml of a scintillation fluid described by Bray (11), and assayed for radioactivity in a Beckman LS-100 spectrometer.

Assay for the Cyclic Trimer - Cyclic $(DBS)_3$ was determined in a similar fashion except that the reaction mixture was diluted with 0.8 ml of a buffer containing 0.5 M KH_2PO_4, pH 6.6, and 0.01 M EDTA prior to the ethyl acetate extraction. Under these conditions, greater than 90% of the neutral cyclic DBS trimer is extracted, and less than 2% of DBS and other acidic DBS containing compounds are extracted. Thus, these procedures offer a convenient assay for cyclic $(DBS)_3$ and other DBS-containing compounds.

Thin-layer chromatography was carried out on MN300 cellulose plates and developed with 5% ammonium formate/0.5% formic acid. DBS and related compounds were located on these plates by UV fluorescence.

Cyclic $(DBS)_3$ was a generous gift of Dr. J.B. Neilands, Department of Biochemistry, University of California, Berkeley, California. O-methyl-DL-serine was purchased from K and K Labs, L-serine methyl ester hydrochloride from Aldrich Chemical Company and N-benzoyl-DL-alanine methyl ester from Mann Research Company.

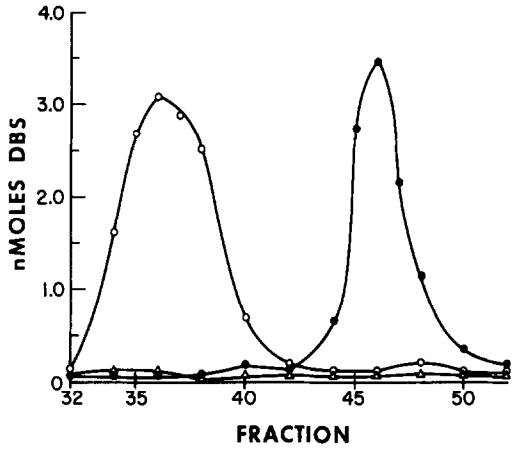

Fig. 2. Sephadex G-100 chromatography of a 0-40% ammonium sulfate fraction. 25 ml of a solution containing 34 mg/ml of protein were applied to an 85 x 5 cm column and 20 ml fractions collected at a flow rate of 20 ml/hr. The column was equilibrated and eluted with a buffer containing: 0.01 M Tris-Cl, pH 7.4, and dithiothreitol, 2.5 mM. 0——0, activity of 50 µl aliquots assayed with 50 µl of fraction 46; ●——●, activity of 50 µl aliquots assayed with 50 µl of fraction 36; △——△, activity of fractions assayed alone.

RESULTS AND DISCUSSION: On the basis of kinetic data a previous report (4) had speculated that more than one enzyme might be involved in the conversion of DBS, L-serine, and ATP to DBS. That this is the case is shown in Fig. 2. When an ammonium sulfate fraction of an active cell-free extract was passed over a Sephadex G-100, and fractions assayed for their ability to synthesize DBS, no activity was observed. However, by combining various column fractions, it was observed that two peaks of activity could be resolved. Fractions 33 to 40, and 44 to 48 were pooled, concentrated by ultrafiltration, and designated E_1 and E_2, respectively.

Requirements for the Reaction - Table I shows that the conversion of L-^{14}C-serine to DBS-containing compounds is completely dependent upon the presence of both E_1 and E_2 as well as DBA and ATP, and that the reaction is stimulated about 3-fold by Mg^{++}. Table I (line 1) also shows that with these purified enzymes about 65% of the DBS-containing material was extractable into ethyl acetate at pH 6.6, and had chromatographic properties similar to those of cyclic $(DBS)_3$.

TABLE I

Requirements for the Enzymatic Synthesis of Total DBS-Containing Compounds and of Cyclic $(DBS)_3$

Omissions	Total DBS-Containing compounds	Cyclic $(DBS)_3$
	nmoles	
None	17.3	11.6
- E_1	0	0
- E_2	0.2	0
- DBA	0.1	0
- ATP	0.1	0.1
- Mg	6.1	2.5

The incubation conditions and the assay are described in the text. Each reaction mixture contained 0.72 mg of E_1 and 0.32 mg of E_2. Products of both assays are expressed in equivalents of DBS.

Fig. 3 shows a chromatogram and the percentage of radioactivity in each of the reaction products after extraction of the incubation mixtures into ethyl acetate from both acid pH (columns 1 and 4) and pH 6.6 (columns 2 and 5). It can be seen (column 1) that when E_1 and E_2 are the source of the enzymes, about 65% of the product is cyclic $(DBS)_3$, about 10% is DBS, and 25% is in the region where the linear trimer (compound II, Fig. 1) and dimer (compound III, Fig. 1) would be expected to chromatograph (6,7). It is to be noted that virtually all of the radioactivity extracted at pH 6.6 (column 2) resides in the cyclic $(DBS)_3$ area. On the other hand, when the ammonium sulfate fraction was the source of the enzymes, all of the radioactivity migrated with the DBS (column 3) and no other ultraviolet fluorescent material was observed.

Thus, these data show that when DBA, serine, and ATP are incubated with an unfractionated enzyme preparation, only DBS is the product while when a more purified enzyme fraction is used, cyclic $(DBS)_3$ is the major product. The

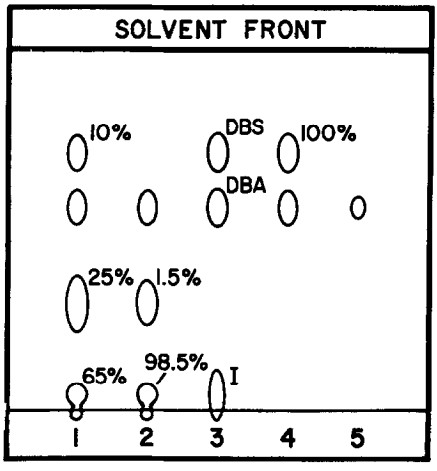

Fig. 3. Thin-layer cellulose chromatography of the products of the reaction in which DBA, L-^{14}C-serine and ATP were incubated with either E_1 and E_2 (column 1 and 2) or 0-40% ammonium sulfate fraction (column 4 and 5). The products were extracted from the reaction mixture at acid pH (column 1 and 4) and at pH 6.6 (column 2 and 5) and after concentrations were chromatographed as described in the text. Column 3 contains standards of cyclic $(DBS)_3$ (I), DBA, and DBS. The outlined areas containing fluorescent spots were scraped off the plates and counted directly in 10 ml of a counting solution as described by Bray (11). The values in parenthesis represent the total radioactivity in percentage found in each fluorescent spot. It is to be noted that the outlined areas only represent the presence of ultraviolet fluorescing material and do not indicate relative amounts.

simplest explanation for these results is that the unfractionated enzyme preparation contains an enzyme which converts cyclic $(DBS)_3$ to DBS, and that this enzyme is only present in limiting amounts in the enzyme preparation which had been purified on Sephadex G-100. That this is the case was verified by incubating the two different enzyme preparations in the presence of cyclic $(DBS)_3$ and analyzing the products of the reaction chromatographically. It was observed that within 5 min the unfractionated enzyme preparation had almost completely hydrolyzed the cyclic $(DBS)_3$ to DBS and other acidic DBS-containing compounds. On the other hand, when fractions E_1 and E_2 were employed, very little cyclic $(DBS)_3$ was hydrolyzed. Other experiments have shown that cyclic $(DBS)_3$ hydrolytic activity was absent from the E_1 fraction and eluted from the Sephadex G-100 column on the trailing edge (Fig. 2) of the E_2 activity.

The above data are consistent with the following sequence of reactions:

$$1)\quad \text{DBA} + \underline{\text{L}}\text{-serine} + \text{ATP} \xrightarrow{E_1 \quad E_2} \text{Cyclic (DBS)}_3$$

$$2)\quad \text{Cyclic (DBS)}_3 \xrightarrow{\text{Hydrolase}} \text{DBS}$$

Since cyclic $(DBS)_3$ contains three equivalents of DBS, it was of interest to ascertain whether DBS was involved as a free intermediate in the synthesis of cyclic $(DBS)_3$. The addition of unlabeled DBS to a incubation reaction containing E_1 and E_2 DBA, $\underline{\text{L}}$-^{14}C-serine, and ATP caused only a slight diminution of the radioactivity found in either cyclic $(DBS)_3$ or DBS. In addition, when tritiated DBS was incubated in the presence of E_1, E_2, ATP, and Mg^{++}, no radioactivity was incorporated into the cyclic $(DBS)_3$ formed. These results suggest that DBS does not occur as a free intermediate in the synthesis of cyclic $(DBS)_3$.

Perhaps the most convincing demonstration that cyclic $(DBS)_3$ is a precursor of DBS, as shown in reactions 1 and 2 above, was illustrated by a trapping experiment in which unlabeled cyclic $(DBS)_3$ was added to an incubation mixture together with an unfractionated enzyme preparation. Reactions were carried out for 5 min with two concentrations of added unlabeled cyclic $(DBS)_3$, and the mixtures assayed for total DBS-containing compounds and for cyclic $(DBS)_3$ synthesis as described above. The total amount of DBS-containing compounds synthesized was constant in all three incubations and equal to 5.03 nmoles. However, at levels of added unlabeled cyclic $(DBS)_3$ of zero, 11.0 and 18.0 nmoles, the amounts of radioactive cyclic $(DBS)_3$ synthesized were 0.07, 1.40 and 2.63 nmoles respectively. These data clearly indicate that cyclic $(DBS)_3$ is an intermediate in the synthesis of DBS.

The specificity of the hydrolase catalyzing reaction 2 above was tested with glycine methyl ester, $\underline{\text{L}}$-serine methyl ester, and N-benzoyl-$\underline{\text{DL}}$-alanine methyl ester, all of which may be regarded as analogues of the ester bonds in cyclic $(DBS)_3$. None of the compounds were hydrolyzed nor did they inhibit the hydrolysis of cyclic $(DBS)_3$. Additional evidence that the enzyme which hydrolyzes cyclic $(DBS)_3$ is highly specific and is very intimately related to the function of the cyclic DBS trimer comes from experiments (Fig. 4) in which it was shown that only extracts of cells which were grown in a low-iron medium (experiment 1) contained

the enzyme which hydrolyzes cyclic $(DBS)_3$. Extracts from cells which were grown in 10 μM iron and which lack the ability to synthesize DBS (10) also cannot hydrolyze cyclic $(DBS)_3$ (experiment 2). In addition, iron-grown cell extracts did not inhibit the activity of the active extracts (experiment 3). Thus, both previous data (10) and these results indicate that the synthesis of the enzymes which synthesize and hydrolyze cyclic $(DBS)_3$ are repressed by iron.

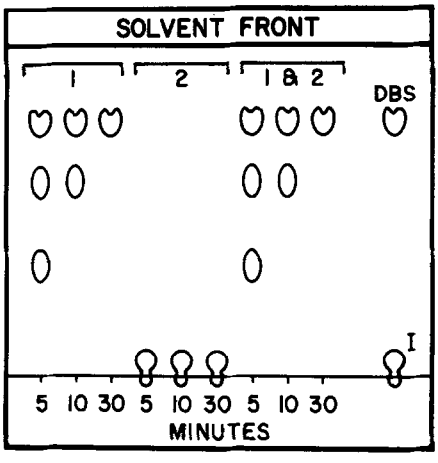

Fig. 4. Thin-layer cellulose chromatography of the products of an incubation containing 0.05 mM cyclic $(DBS)_3$ in 0.1 M Tris-buffer, pH 8.0, with a crude cell-free extract (about 1 mg protein) of low-iron grown cells, (1) and cells repressed by growth in 10 μM iron, (2). Compounds were detected by UV fluorescence. The standards seen on the right are cyclic $(DBS)_3$ (I) and DBS. The intermediate spots are the breakdown products of cyclic $(DBS)_3$ noted previously.

Cyclic $(DBS)_3$ provides a very compact binding site for iron and the complex with the ferric form probably has a very high stability, perhaps comparable to the ferrioxamines and ferrichromes. In order for the molecule to function as an iron transport system, a means must be present for the release of the sequestered metal ion when it is carried into the cell. Direct transfer to an acceptor is probably unlikely on thermodynamic and accessibility grounds. It is suggested that the function of the cyclic $(DBS)_3$ hydrolase, which causes the hydrolysis of the iron-containing cyclic $(DBS)_3$ complex, is to convert the iron to a less tightly bound form which is then transferred to other cellular components.

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