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Biosynthesis of 7,8-Diaminopelargonic Acid, a Biotin Intermediate, from 7-Keto-8-Aminopelargonic Acid and *S*-Adenosyl-L-Methionine[1]

MAX A. EISENBERG AND GERALD L. STONER[2]

Department of Biochemistry, College of Physicians & Surgeons, Columbia University, New York, New York 10032

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Resting cells of *Escherichia coli* strain D302(*bioD*302) can synthesize 7,8-diaminopelargonic acid from 7-keto-8-aminopelargonic acid. The product of this aminotransferase reaction has been identified by paper chromatography and electrophoresis. Glucose enhances the vitamer yield twofold. Of the 19 amino acids tested as amino donors, only methionine proved to be significantly stimulatory. In cell-free extracts, however, methionine was completely inactive unless both adenosine triphosphate (ATP) and Mg^{2+} were present. *S*-Adenosyl-L-methionine (SAM) was about 10 times more effective than methionine, ATP, and Mg^{2+}. The optimal conditions for the reaction were determined, and substrate inhibition was found for 7-keto-8-aminopelargonic acid. It has been possible to eliminate certain impurities as amino donors in the commercial preparation of SAM and those that may arise in enzymatic reactions in which SAM is a substrate. The direct participation of SAM in the aminotransferase reaction seems a likely possibility.

The following scheme for the biosynthesis of biotin was proposed by Rolfe and Eisenberg (12): $\xrightarrow[C]{}$ pimelic coenzyme A + alanine $\xrightarrow[F,G]{I}$ 7-keto-8-aminopelargonic acid $\xrightarrow[A]{II}$ 7,8-diaminopelargonic acid $\xrightarrow[D]{III}$ dethiobiotin $\xrightarrow[B]{IV}$ biotin. The earlier evidence rested primarily on the results of cross-feeding experiments and on the excretion patterns of 60 biotin auxotrophs isolated from *Escherichia coli* K-12 strain Y-10, which could be divided into four mutant groups. A similar scheme was also proposed by Pai (8). Preliminary genetic mapping of the four mutant groups was carried out by Del Campillo-Campbell et al. (1) and Rolfe and Eisenberg (12). As the result of fine-structure mapping and complementation analysis, Rolfe (11) was able to divide the mutants into seven complementation groups and define the biotin operon as follows: λ *att A B E F G C D*. The particular steps which these genes control in the above reaction sequence are indicated by capital letters under the arrows.

Studies with cell-free extracts and purified enzyme systems have firmly established the product-precursor relationships of reactions I (2) and III (5, 7). The present study provides evidence for the biosynthesis of 7,8-diaminopelargonic acid (DAPA) from 7-keto-8-aminopelargonic acid (7-KAP, reaction II) in both resting-cell suspensions and cell-free extracts. While this investigation was in progress, Pai (9), reporting on the same reaction in a cell-free system, found methionine to be the amino donor. The enzymatic activity was determined by coupling the reaction with the dethiobiotin synthetase enzyme and assaying for dethiobiotin. However, our evidence presented in this paper suggests the active participation of *S*-adenosyl-L-methionine (SAM) rather than methionine.

MATERIALS AND METHODS

The preparation of 7-KAP and DAPA have been described in a previous communication (12). S. Okumura of the Ajinomoto Co. generously supplied a sample of crystalline 8-keto-7-aminopelargonic acid (8-KAP) and a partially purified preparation of 7,8-diketopelargonic acid. The SAM, L-methionine, and tris-(hydroxymethyl)aminomethane (Tris, enzyme and buffer grade) were products of Schwartz/Mann. DL-homocysteine, free base, was obtained from Nutritional Biochemicals Corp. SAM [molar absorbancy (E_m), 260 nm = 15,400 liters $\times$ mol^{-1} $\times$ cm^{-1}] was also pre-

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pared from yeast (14) and purified by the method of Pegg and Williams-Ashman (10). Adenosine triphosphate (ATP), pyridoxal-5-phosphate (PLP), *S*-adenosyl-L-homocysteine, L-homoserine, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

Ascending paper chromatography and paper electrophoresis were done as previously described (12). The solvent systems and buffers are described in the footnotes to the tables. Chromagrams Eastman Organic Chemicals were used with solvent system 1 for determining the purity of SAM.

A biotin auxotroph of *E. coli* K-12, strain D302-(*bioD*), was used for the resting-cell and the early cell-free extract studies. This mutant lacks the enzyme dethiobiotin synthetase and accumulates 7-KAP and DAPA in the growth medium. The growth conditions as well as the method for derepression have been described previously (5). Strain R136(*bioR*) was subsequently substituted for the above strain since it is fully derepressed as the result of a mutation in the repressor gene. This mutant excretes about 100 times more biotin vitamers than does the parent strain, AB313. The method of its isolation and mapping will be described in a subsequent report.

For the preparation of the cell-free extracts, strain R136 was grown overnight in 1 liter of 1% tryptone broth (Difco) which was used as an inoculum for 50 liters of the same medium contained in a model F-130 fermentor (New Brunswick Scientific Co.). The cells were grown with continuous stirring (200 rev/min) and aeration (5 liters/min) to an optical density of 200 Klett units before harvesting by centrifugation. The cells were washed once in saline, suspended in 0.05 M Tris buffer (*p*H 7.5) containing 0.01 M mercaptoethanol, and broken by sonic disintegration (Branson model W140D). The mixture was centrifuged at 30,000 rev/min for 30 min in a type 30 rotor of the Beckman model L ultracentrifuge, and the supernatant fraction was dialyzed against two changes of the same buffer.

The enzymatic assay in cell-free extracts, except where indicated, contained the following components in a final volume of 0.5 ml: 150 mM Tris buffer (*p*H 8.5), 0.01 mM 7-KAP, 2.5 mM SAM, 0.2 mM PLP, 5 mM DTT, and 0.5 to 1 mg of protein. The enzyme, buffer, and DTT were preincubated for 10 min at 37 C and then a mixture of both substrates and cofactor in buffer was added to start the reaction. After 30 min, the reaction was terminated by the addition of 0.2 ml of 12% trichloroacetic acid. The disc assay method was used to quantitate the product of the reaction (3). Strain A109(*bioA*) which responds more readily to DAPA than does any other *bioA* mutant was the assay organism of choice.

RESULTS

Resting-cell suspensions. When a buffered, resting-cell suspension of strain D302 was incubated with 7-KAP as the only substrate, a biotin vitamer was produced that would support the growth of strain A109, which can grow on DAPA in place of biotin but cannot grow on 7-KAP. This reaction could be demonstrated in resting-cell suspensions of all mutant groups except *bioA* and the mutant T_{60-1} which lacks the entire biotin operon, indicating that this reaction is specific for the biotin pathway and is not a generalized aminotransferase reaction. A study of the optimal conditions for this reaction showed enhanced vitamer yield with increasing concentrations of 7-KAP up to 0.01 mM and a subsequent decrease with further increases in substrate concentration. The addition of glucose usually produced a twofold increase in vitamer yield. Glutamic and aspartic acids, which are active amino donors in many aminotransferase reactions, had little effect on the reaction. In search for other possible amino donors, 19 amino acids were tested. The results shown in Table 1 indicate that methionine gave a twofold stimulation in vitamer production. However, the methionine effect was reduced 50% if glucose was omitted from the reaction mixture.

Cell-free extracts. To study the reaction mechanism in greater detail, we turned our attention to a cell-free system. When 7-KAP, L-methionine, and PLP were incubated together with a cell-free extract for a period of 1 hr, no vitamer could be detected with strain A109. Altering the concentration of the L-methionine and the *p*H of the reaction mixture was without effect. Since the resting cells responded to added glucose, the possibility of an energy source had to be considered. When ATP and Mg^{2+} were added to the above reaction mixture, formation of a vitamer could be readily detected with strain A109. The inability of methionine to function without the addition of ATP suggested the activation of

TABLE 1. *Amino donors for 7,8-diaminopelargonic acid synthesis in resting cells of strain D302*[a]

Addition	Nanomoles per milliliter	Addition	Nanomoles per milliliter
None	0.31	Threonine	0.34
Methionine	0.62	Histidine	0.34
Aspartic acid	0.46	Phenylalanine	0.34
Asparagine	0.40	Arginine	0.31
Tyrosine	0.39	Glutamic acid	0.31
Cysteine	0.37	Lysine	0.31
Proline	0.37	Tryptophan	0.31
Serine	0.37	Valine	0.31
Glycine	0.34	Leucine	0.29
Glutamine	0.34	Alanine	0.26

[a] Reaction mixture in a final volume of 1.0 ml contained 150 mM phosphate buffer (*p*H 7.5), 1% glucose, 0.01 mM 7-keto-8-aminopelargonic acid, 5 mM L amino acids, and 6 mg of cells (dry weight). Incubation was for 1 hr at 37 C. The mixture was cooled in ice and centrifuged.

some component of the system. The formation of SAM from L-methionine and ATP in the crude enzyme preparation appeared to be a likely possibility (16). The results shown in Table 2 indicate that, as expected, SAM replaced the requirement for L-methionine and ATP. In the dialyzed cell-free system, optimal concentrations of SAM gave a 10-fold higher yield of product than optimal concentrations of L-methionine, ATP, and Mg^{2+}. When pyridoxal phosphate was omitted from the reaction mixture, the yield was reduced by 50%. Complete dependence on the cofactor even in extensively dialyzed preparations could not be demonstrated.

The product of the reaction with either SAM or L-methionine, ATP, and Mg^{2+} was identified as DAPA by comparing its electrophoretic and chromatographic behavior with an authentic sample of DAPA as shown in Table 3.

Since SAM can enter into a large number of enzymatic reactions, the possibility that it was not a direct participant in the aminotransferase reaction observed with the crude extract had to be considered. We therefore tried homoserine, homocysteine, *S*-adenosyl-homocysteine, and spermine in the routine reaction mixture in place of SAM and found no evidence of DAPA formation. These amino compounds are end products in a variety of reactions in which SAM is a substrate. In addition, the commercial preparation of SAM, reported as 80% pure, showed six ultraviolet (UV)-absorbing components on thin-layer chromatography in solvent system 1. Of the two major components, one was also ninhydrin positive and had an R_F value which corresponded to the reported value of 0.18 for SAM (15). The other major component corresponded to 5'-methylthioadenosine. The six UV-absorbing regions were punched out of the chromatograms with a paper punch, and the Silica Gel discs were

TABLE 2. *7,8-Diaminopelargonic acid synthesis in dialyzed cell-free extracts of strain D302*[a]

Additions	Nanomoles
Methionine, ATP, Mg^{2+}	0.09
Methionine, Mg^{2+}	0.00
Methionine, ATP	0.00
SAM	1.05

[a] Reaction mixture in a volume of 0.5 ml contained: 100 mM tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 8.0), 0.01 mM 7-keto-8-aminopelargonic acid, 0.2 mM pyridoxal-5-phosphate, 1.0 mM *S*-adenosyl-L-methionine (SAM), 10 mM each adenosine triphosphate (ATP), Mg^{2+}, and L-methionine, and 2.44 mg of enzyme. The reaction mixture was incubated for 1 hr at 37 C and was terminated by adding 0.2 ml of 12% trichloroacetic acid.

TABLE 3. *Identification of product by chromatography and electrophoresis*

Component	R_F[a]		Mobility[b]			
	Solvent 1	Solvent 2	*p*H 2	*p*H 3	*p*H 5	*p*H 7
Product	0.42	0.32	−10.8	−9.3	−6.6	−1.3
DAPA[c]	0.43	0.33	−10.9	−9.2	−6.7	−1.6

[a] Solvent 1, 1-butanol-glacial acetic acid-water (60:15:25); solvent 2, 1-butanol-formic acid-water (4:1:1).

[b] Buffers: *p*H 2, 0.6 M acetic and 0.1 M formic acids; *p*H 3, 0.025 M sodium citrate; *p*H 5, 0.025 M sodium acetate; *p*H 7, 0.025 M sodium phosphate.

[c] 7,8-Diaminopelargonic acid.

placed onto individual 6-mm paper assay discs to which was added 20 μliters of the remaining components of the reaction mixture. After incubating for 1 hr, the discs were placed onto the surface of the assay plate. Only one of the compounds, SAM, gave rise to DAPA. The commercial preparation was purified by column chromatography. A fresh preparation of SAM made from yeast was purified by the same procedure. Both preparations assayed over 90% pure on the basis of UV absorption, and both were equally effective in the assay.

The optimal conditions for DAPA formation with the dialyzed cell-free extracts are shown in Fig. 1. The reaction is linear with time up to about 40 min and also is proportional to protein concentration up to about 3 mg per ml of reaction mixture. The saturating concentration for SAM is about 2 mM and the *p*H optimum for the reaction is 8.5. Although phosphate buffer is slightly less effective than Tris buffer at lower *p*H values, no inhibition was observed when phosphate was added to the Tris buffer. 7-KAP stimulates DAPA formation at concentrations lower than 0.015 mM and inhibits at higher concentration (Fig. 2). Almost identical results were also obtained with the resting-cell suspensions. At the optimal concentration, approximately 20% of the 7-KAP is converted into DAPA. The addition of 8-KAP, an isomer of 7-KAP, also results in DAPA formation but does not show the substrate inhibition (Fig. 2). It saturates the system at about 60 times the concentration at which 7-KAP does and produces only about one-fifth the amount of DAPA under the same conditions. We have also observed the formation of DAPA from 7,8-diketoperlargonic acid but have not been able to quantitate its activity because of the impurity of the sample.

It was observed that the dialysis of the crude extract invariably resulted in the loss of approximately 50% of the enzymatic activity. Attempts to restore the activity by adding back various

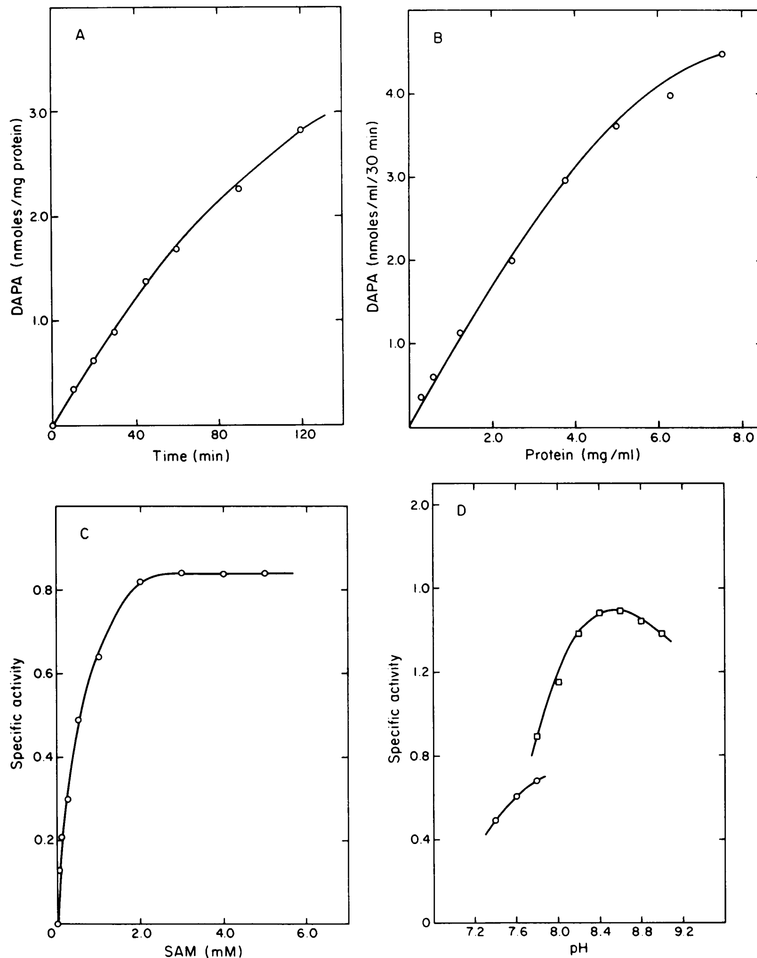

FIG. 1. *Optimal conditions for aminotransferase reaction. The reaction mixture, containing 0.63 mg of protein of cell-free extract of strain R136, and the conditions were as described in Materials and Methods except where variable (A) as a function of time, (B) as a function of protein concentration, (C) as a function of SAM concentration, (D) as a function of pH; ⊙, phosphate buffer, □, Tris buffer, both 150 mM. Specific activity is expressed as nanomoles of DAPA formed per milligram of protein in 30 min.*

cofactors and the supernatant fraction from the boiled crude enzyme preparation were without success. It was subsequently found that concentrations of 2-mercaptoethanol in the reaction mixture higher than that normally introduced with the dialyzed enzyme markedly stimulated the reaction, suggesting a requirement for free sulfhydryl groups for optimal enzyme activity. A study of a number of sulfhydryl reagents indicated that dithiothreitol was more effective than mercaptoethanol, glutathione, or cysteine in restoring enzymatic activity. The most effective procedure for obtaining maximal activity was to preincubate the enzyme in buffer and DTT for 10 min prior to the addition of substrates and cofactor.

DISCUSSION

The present study provides additional evidence to support an intermediary role for DAPA in the biosynthetic pathway for biotin and confirms the results of Pai (9). The members of the mutant group classified as *bioA* lack the aminotransferase enzyme. The reason that methionine was proposed as the amino donor by Pai (9) can be attributed to the use of a coupling system in which purified dethiobiotin synthetase was added along with the necessary substrates, ATP and $NaHCO_3$, to convert the DAPA into dethiobiotin. This was necessitated because of the insensitivity of his assay organism to DAPA. Therefore, the presence of both ATP and methionine in the coupled system resulted in the formation

of SAM (16). With the aid of a more sensitive strain, we have been able to detect and measure the end product of the reaction directly. The isolate from strain A109 used in these experiments can grow readily on as little as 0.5 ng of DAPA per ml as compared to 0.1 ng of biotin per ml. We have followed the enzymatic activity catalyzing the formation of DAPA from 7-KAP, methionine, and ATP through preliminary purification procedures and have obtained fractions from a diethylaminoethyl cellulose column which are inactive with methionine and ATP but are active with SAM.

The inhibition of DAPA synthesis by high concentrations of 7-KAP was observed in both resting cells and cell-free extracts, corroborating the finding of Pai (9). The dialyzed extracts are also capable of acting upon 8-KAP and the diketo analogue, both of which have been previously shown by Okumura et al. (6) to support the growth of biotin auxotrophs of *Brevibacterium*. In their scheme for biotin biosynthesis, Okumura et al. suggested the diketo analogue as the precursor of 7-KAP. However, we have not been able to detect diketopelargonic acid in the filtrates of *bioA* mutants. It will be of interest to see if 7-KAP or 8-KAP is the intermediate in the synthesis of DAPA from the diketo compound.

SAM has been shown to be involved as a methyl donor in a variety of transmethylation reactions. It is also known that after decarboxylation SAM can serve as a donor of the propylamine moiety in spermidine and spermine biosynthesis (10). In methionine biosynthesis, SAM serves as an activator of the enzyme without participating directly in the reaction (13). To our knowledge, this is the first instance of SAM being involved in an aminotransferase reaction. We have been able to eliminate certain amino donors which are end products of reactions involving SAM as a substrate. Similarly, the contaminants present in the commercial preparations of SAM have also been excluded. If SAM is the immediate amino donor, then its keto analogue should accumulate in the reaction mixture. This compound should also have UV-absorbing properties and possibly could be detected by its ability to absorb UV light. The reaction mixture with and without 7-KAP was scaled up fourfold, concentrated, and separated on thin-layer chromatograms in solvent system 1. No additional UV-absorbing compound could be detected in the complete reaction mixture. The difficulty in this approach is the limitation on the yield of the end product imposed by the substrate inhibition of 7-KAP. The identification of the second product of the reaction will be facilitated when further purification of the enzyme eliminates side

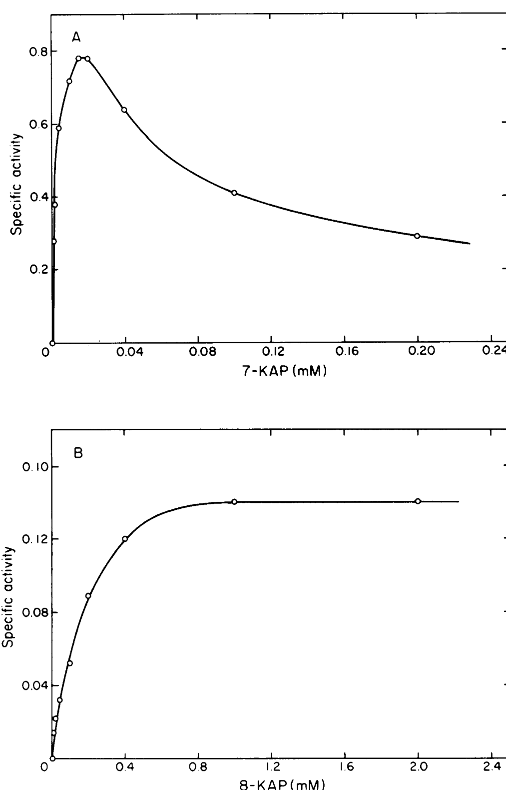

FIG. 2. *Effect of 7-KAP (A) and 8-KAP (B) concentrations on aminotransferase activity. The concentrations of the other components of the reaction mixture were as described in Materials and Methods, and the protein concentration is the same as given in the legend of Fig. 1. Specific activity is expressed as nanomoles of DAPA formed per milligram protein in 30 min.*

reactions involving SAM. Despite the lack of information concerning the mechanism of this reaction, a sensitive assay for the product of cistron A is now available. This assay is of particular interest in light of the model for the control of the biotin operon recently proposed by Guha et al. (4). Based on experiments in which messenger ribonucleic acid transcribed from the biotin operon was hybridized with DNA from lambda phages carrying particular regions of the biotin locus, Guha et al. proposed a divergent transcription of the biotin operon, with cistron A transcribed to the left on the *l* strand and cistrons B thru D transcribed to the right on the *r* strand. The operators were placed between cistrons A and B. Cistron B, which may be multicistronic, codes for the enzyme or enzymes catalyzing the incorporation of sulfur (reaction IV), whereas cistron A codes for the aminotransferase

enzyme (reaction II). The assay for the product of the A cistron reported herein provides the means for a biochemical verification of this unique model for control. This is now in progress.

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