Ferredoxin-dependent Phenylpyruvate Synthesis by Cell-free Preparations of Photosynthetic Bacteria*

ULRICH GEHRING and DANIEL I. ARNON
From the Department of Cell Physiology, University of California, Berkeley, California 94720

SUMMARY

A reductive synthesis of phenylpyruvate from phenylacetyl coenzyme A and bicarbonate was obtained in cell-free extracts from the photosynthetic bacterium Chromatium. This phenylpyruvate synthase activity—which appears to be distinct from pyruvate synthase—depends on the reducing power of ferredoxin and requires thiamine pyrophosphate as a coenzyme. Of several ferredoxins assayed, the native ferredoxin from Chromatium was found to be the most effective for phenylpyruvate synthesis.

The enzyme extract also catalyzed the conversion of phenylpyruvate to phenylalanine (in the presence of an amino group donor) and the activation of phenylacetate to the phenylacetyl-CoA derivative. Thus, a net synthesis of phenylalanine from phenylacetate and CO₂ was accomplished.

Closely associated with the phenylpyruvate synthase reaction was an exchange reaction between ¹⁴C-bicarbonate and phenylpyruvate. The exchange reaction required thiamine pyrophosphate and CoA as cofactors.

Apart from Chromatium, phenylpyruvate synthase and phenylpyruvate-bicarbonate exchange activities were also found in cell-free extracts from the green photosynthetic bacteria Chlorobium thiosulfatophilum and Chloropseudomonas ethylicum.

Previous work in this laboratory has revealed a direct participation of reduced ferredoxin in CO₂ fixation reactions in bacterial photosynthesis. These reactions, driven by the strongly reducing potential of ferredoxin—about equal to that of molecular hydrogen (1, 2)—involve a reductive carboxylation of an acyl coenzyme A derivative to form the corresponding α-keto acid (Equation 1).

R-CH₂-CO₂H + ferrodoxin → R-CH₂-CO₂H + ferrodoxin + CO₂

The first such reaction in photosynthetic bacteria was the synthesis of pyruvate from CO₂ and acetyl-CoA found by Buchanan, Bachofen, and Arnon in Chromatium (3). Pyruvate synthase is now known to be present in various types of photosynthetic (4-6) and nonphotosynthetic anaerobic bacteria (7-12).

A second reaction of this type is the reductive carboxylation of succinyl-CoA to form α-ketoglutarate, which has been found in certain photosynthetic bacteria (4, 6, 13) and has also been observed recently in an anaerobic rumen bacterium (14). Another ferredoxin-dependent reductive carboxylation reaction is the synthesis of α-ketoglutarate from CO₂ and propionyl-CoA discovered recently by Buchanan (15). Pyruvate synthase and α-ketoglutarate synthase are key enzymes in the reductive carboxylic acid cycle (16, 17), a new cyclic pathway for CO₂ fixation in bacterial photosynthesis.

A common feature of the three ferredoxin-dependent reductive carboxylation reactions is that the α-keto acids formed are related to the biosynthesis of amino acids which, in photosynthetic bacteria, constitute the main soluble product of CO₂ fixation (18-22). Thus, when Allison and Robinson (23, 24) found that ¹⁴C-carboxyl-labeled phenylacetate was recovered from cellular protein as ²⁴C-phenylalanine, it seemed desirable to investigate whether these observations also involved an initial reductive carboxylation of phenylacetyl-CoA to phenylpyruvate and its subsequent amination to phenylalanine.

This paper presents evidence for a ferredoxin-dependent reductive carboxylation of phenylacetyl-CoA to phenylpyruvate, in accordance with Equation 2.

Phenylacetyl-CoA + CO₂ + ferrodoxin → phenylpyruvate + CoA + ferrodoxin

This enzyme system, which will be called phenylpyruvate synthase, was found in cell-free preparations of the photosynthetic bacteria Chromatium, C. thiosulfatophilum, and C. ethylicum.

METHODS

Chromatium, strain I, was grown in 13-liter Pyrex bottles either in the bicarbonate-malate medium of Arnon, Das, and Anderson (21) or in the same medium modified by replacing malate with sodium thiosulfate (final concentration 0.012 M). C. thiosulfatophilum, strain Tassajara, was grown as described by Evans and Buchanan (3) in a medium free of acetate. C. ethylicum, kindly supplied by Dr. J. M. Olson of the Brookhaven National Laboratory, was cultured as described by Evans (6). The harvested cells were stored at −20°C prior to use.

The enzyme preparation was freshly made (at 4°C under argon or oxygen-free N₂) for each experiment from 10 g of cell paste.
that was thawed, suspended in 10 ml of 0.02 M HEPES* buffer, pH 7.7 (containing 100 μmoles of diethiothreitol), and disrupted by sonication for 2 min with a Branson sonifier (power setting 6). Buffer solutions were rendered aneurable by bubbling argon gas.

The sonified cell suspension was centrifuged for 10 min at 30,000 × g and the precipitate was discarded. Ferredoxin was removed from the supernatant fluid by passing it through a DEAE-cellulose column (2 × 3 cm) equilibrated with 0.02 M HEPES buffer, pH 7.7. An effluent volume about equal to the volume applied was collected and centrifuged for 2 hours at about 100,000 × g, and the precipitate was discarded. To remove possible interfering substrates, the supernatant fluid was passed through a Sephadex G-25 column (2 × 36 cm) equilibrated with 0.02 M HEPES buffer, pH 7.7. The excluded protein fraction was used as the enzyme preparation. The protein content was estimated by a modified Bieuret method (25) in which the Folin procedure (30) with crystalline bovine serum albumin was used as standard.

The ferredoxins were reduced photochemically by heated spinach chloroplast fragments (31) as described by Buchen, Buchanan, and Arnon (7).

Phenylacetyl-CoA was prepared from phenylacetic anhydride by the acid anhydride method of Simon and Shenin (32) (on paper chromatography (33) with a 1:1 mixture of ethanol and 0.1 M sodium acetate buffer, pH 4.5, phenylacetyl-CoA gave a slightly lower Rf value (0.74 at 25°) than acetyl-CoA). Phenylactic anhydride was synthesized from the free acid by boiling with excess acetic anhydride (34) and recrystallized twice from benzene-petroleum ether.

The newly formed 14C-phenylpyruvate was determined as the 2,4-dinitrophenylhydrazone after adding unlabeled sodium phenylpyruvate as carrier. The 14C-labeled 2,4-dinitrophenylhydrazone was identified by paper chromatography (solvent systems, 1-butanol-2 × NH4-ethanol, 7:2:1 (35); tertiery amyl alcohol-ethanol-water, 5:1:4 (36)) and subsequent autoradiography. Labeled phenylalanine was identified and separated from phenylpyruvate by the thin layer chromatography method (slightly modified) described by Buchan, Buchanan, and Arnon (15). The identity of the labeled compound with authentic phenylalanine was established by the coincidence of the radioactive spot with the spot of authentic phenylalanine as stained with ninhydrin. For the quantitative estimation of phenylalanine the radioactive areas of duplicate chromatograms were scraped from the plates, and the material was mixed with scintillation fluid (37) and counted in a scintillation counter.

RESULTS AND DISCUSSION

Synthesis of Phenylpyruvate—The requirements for phenylpyruvate synthesis by an enzyme preparation from Chromatium cells, grown autotrophically in a bicarbonate-thiosulfate medium, are shown in Table I. The reaction mixture contained either

1 The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

| Treatment | From HPCO3- (Experiment A) | From 14C-Phenylacetate (Experiment B) |
|-----------|----------------------------|----------------------------------------|
| Complete  | 13,350                     | 30,800                                  |
| Phenylacetate omitted | 50                        |                                        |
| Ferredoxin omitted     | 900                       | 950                                    |
| ATP omitted            | 2,200                     | 3,300                                  |
| MgCl2 omitted          | 2,400                     | 4,400                                  |
| Coenzyme A omitted     | 1,600                     | 500                                    |
| Thiamine pyrophosphate omitted | 1,700              |                                         |
| Enzyme preparation omitted | 80                      | 100                                    |
| Complete, ferredoxin not reduced | 0                        | 0                                      |

14C-bicarbonate and unlabeled phenylacetate (Experiment A) or 14C-phenylacetate and unlabeled bicarbonate (Experiment B). Formation of phenylpyruvate occurred only in the presence of reduced ferredoxin (photoreduced by chloroplasts) and, aside from phenylacetate and 14CO3-, was dependent on thiamine pyrophosphate, ATP, CoA, and Mg++. In the complete system, phenylpyruvate was formed at a rate of about 270 nanomoles per hr per 10 mg of crude enzyme.

The enzyme preparation from Chromatium contained an acyl-CoA synthetase that catalyzed the conversion of free phenylacetate to the CoA derivative in the presence of CoA, ATP, and Mg++. (Treatments 1, 2, and 3, Table II). Synthetic phenylacetyl-CoA was also effective in the reaction (Treatment 4, Table II); however, it did not give as good an incorporation of 14CO2 as the phenylacetyl-CoA generated in situ, possibly because the relatively high concentration of the synthetic compound was somewhat inhibitory. When phenylacetyl-CoA was generated in the reaction mixture, the rate of phenylpyruvate synthesis was about the same, whether all of the ATP was added initially or whether it was being generated by the creatine phosphate-phosphokinase couple (Treatments 3 and 5, Table II). In contrast to the pyruvate synthase (3) and α-ketobutyrate synthase (15) systems, the addition of semicarbazide as a carbonyl trapping agent did not increase the synthesis of phenylpyruvate.

The formation of phenylpyruvate, as measured under the conditions described in Table I, proceeded linearly for about 70 min and was proportional to the amount of enzyme preparation added.

---

*The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
TABLE II
Comparison of exogenous and nascent phenylacetoyl-CoA in synthesis of phenylpyruvate

Experimental conditions were as in Experiment A of Table I except that, where indicated, (a) synthetic phenylacetoyl-CoA (3.5 μmoles) replaced phenylacetate, CoA, ATP, and Mg ++, and (b) ATP was omitted, replaced by ADP (5 μmoles), or generated from creatine phosphate (30 μmoles) and ADP (1 μmole) in the presence of creatine phosphokinase (0.1 mg).

| Treatment | 14C-Phenylpyruvate formed cpm |
|-----------|--------------------------------|
| 1. Phenylacetate + CoA | 50 |
| 2. Phenylacetate + CoA + ADP | 500 |
| 3. Phenylacetate + CoA + ATP | 14,700 |
| 4. Synthetic phenylacetoyl-CoA | 6,300 |
| 5. Phenylacetate + CoA + ATP generated | 15,600 |

FIG. 1. Differences in the pH profiles of the phenylpyruvate synthase and phenylpyruvate-bicarbonate exchange reactions. Experimental conditions for the phenylpyruvate synthase were as described for Experiment A in Table I and for the exchange reaction as given for the complete treatment in Table VI.

TABLE III
Effect of ferredoxins from different sources on synthesis of phenylpyruvate

Each ferredoxin (250 μg) was added as indicated; other experimental conditions were as given for Experiment A of Table I.

| Source of added ferredoxin | 14C-Phenylpyruvate formed cpm |
|---------------------------|--------------------------------|
| None added | 130 |
| Chromatium | 15,700 |
| Chlorobium thiosulfatophilum | 11,700 |
| Azotobacter vinelandii | 7,400 |
| Clostridium pasteurianum | 4,700 |
| Spinach | 400 |

Fig. 2. Effect of ferredoxin concentration on synthesis of phenylpyruvate. Experimental conditions were as described for Experiment A in Table I except that the ferredoxins from Chromatium and Clostridium pasteurianum were varied as indicated.

TABLE IV
Occurrence of phenylpyruvate synthase in extracts of Chromatium cells grown in inorganic and organic media

Experimental conditions were as described for Experiment A of Table I.

| Culture medium          | 14C-Phenylpyruvate formed cpm |
|-------------------------|--------------------------------|
| Bicarbonate-thiosulfate |                                |
| Lot A                   | 13,350                         |
| Lot B                   | 9,200                          |
| Bicarbonate-malate      |                                |
| Lot C                   | 11,400                         |
| Lot D                   | 8,600                          |

up to about 12 mg of protein per 3 ml of reaction mixture. The optimum pH range for phenylpyruvate synthesis was 8.1 to 8.6 (Fig. 1), whereas the optimum for the phenylpyruvate-bicarbonate exchange, which is discussed below, was pH 7.2.

A comparison of the relative effectiveness of different ferredoxins in the phenylpyruvate synthase reaction showed that the ferredoxins from the two photosynthetic bacteria Chromatium and C. thiosulfatophilum were the most effective (Table III). Clostridium ferredoxin was considerably less active, and the effectiveness of the recently isolated new type of ferredoxin from A. vinelandii was intermediate between that of Chromatium and Clostridium. Spinach ferredoxin, known to be a poor substitute for bacterial ferredoxins in the phosphorolactic cleavage of pyruvate and in the nitrogenase system of C. pasteurianum, was also virtually without effect in the phenylpyruvate synthase reaction.

The concentration effect of ferredoxin is shown in Fig. 2. About 180 μg (per 3 ml) of Chromatium ferredoxin saturated the phenylpyruvate synthase system; Clostridium ferredoxin failed to saturate, even at a concentration of 400 μg per 3 ml. A Lineweaver-Burk plot of the data showed the same Vmax for both ferredoxins and gave an apparent Km of 5 × 10⁻⁷ M for Chromatium ferredoxin and 8 × 10⁻⁴ M for Clostridium ferredoxin.

The experiments described so far were carried out with enzyme preparations from Chromatium cells grown autotrophically in a bicarbonate-thiosulfate medium. Table IV shows that comparable levels of phenylpyruvate synthase activity were also present in extracts of cells grown in a malate medium—an observation.
that is compatible with the report of Allison and Robinson (23) of equal uptake of "C-phenylacetate by Chromatium cells grown in either a bicarbonate-thiosulfate or a malate medium. Phenylpyruvate synthase appears to be a constitutive rather than an inducible enzyme of Chromatium cells. Its formation was not affected by the addition of phenylacetate (1 mM) to the culture medium.

**Synthesis of Phenylalanine from Phenylacetate**—The addition of glutamate or glutamine to the reaction mixture did not alter substantially the total 14C-bicarbonate fixed but changed drastically the final products formed from phenylacetate (Table V). A sharp decrease in 14C-phenylpyruvate was accompanied by a marked increase in 14C-phenylalanine. These results show a net cell-free synthesis of phenylalanine from phenylacetate and CO2. In the presence of either glutamate or glutamine there was also some incorporation of 14C-bicarbonate that could not be accounted for by the methods used.

**Phenylpyruvate-Bicarbonate Exchange**—Enzyme preparations from Chromatium cells were found to catalyze a 14C exchange reaction between 14C-bicarbonate and phenylpyruvate. Incorporation of 14C into phenylpyruvate occurred only when thymine pyrophosphate and catalytic amounts of CoA were present in the reaction mixture (Table VI). CoA could not be replaced by any of the other sulfhydryl compounds tested. However, a vigorous exchange occurred without added CoA when the system was illuminated in the presence of chloroplasts, i.e., under conditions that could have brought about the photoreduction of any ferredoxin present. Although no ferredoxin was added in this case, it seems likely that the activation of the exchange reaction by illumination was due to the reduction of trace amounts of ferredoxin that remained in the enzyme preparation after it was passed through the DEAE-cellulose column. The possibility that reduced ferredoxin did indeed replace CoA was strengthened by the observed stimulation of the exchange reaction upon adding ferredoxin to the illuminated reaction mixture. Added NADH or NADPH was wholly ineffective as a substitute for CoA but some exchange activity occurred in the presence of sodium dithionite, possibly due to the reduction of traces of ferredoxin that remained in the reaction mixture.

The pH curve for the CoA-catalyzed exchange reaction had a relatively flat drop on the acid side of the optimum pH of 7.2 (Fig. I)—a distinctly different pH profile from that of the phenylpyruvate synthase reaction. At their respective pH optima, the rate of the exchange reaction was about three times greater than the rate of phenylpyruvate synthase.

**Phenylpyruvate Synthase Versus Pyruvate Synthase**—Phenylpyruvate synthase appears to be distinct from pyruvate synthase. This view is supported by (a) the presence of the pyruvate but not the phenylpyruvate enzyme in C. pasteurianum (see below), (b) differences in stability of phenylpyruvate synthase and pyruvate synthase in Chromatium cells stored at -20° for 4 months of storage, some cells had no phenylpyruvate synthase activity while retaining a high level of pyruvate synthase activity, and (c) differential requirements of thiamine pyrophosphate. Phenylpyruvate synthase by cell-free extracts of Chromatium had a distinct requirement for thiamine pyrophosphate (Table I). By contrast, pyruvate synthase of Chromatium shows such a requirement only after the endogenous thiamine pyrophosphate is removed by special treatments (38).

**Phenylpyruvate Synthase in Other Organisms**—Aside from Chromatium, comparable phenylpyruvate synthase activity was found in cell-free extracts of the green photosynthetic bacteria *C. thiosulfatophilum* and *C. ethlicum* (assayed as described for Experiment A in Table I). These cell-free extracts also showed phenylpyruvate-bicarbonate exchange activity. Thus, the phenylpyruvate-bicarbonate exchange activity appears to be always closely associated with phenylpyruvate synthase. Neither phenylpyruvate synthase nor phenylpyruvate-bicarbonate exchange activity was detected in cell-free extracts of the non-photosynthetic anaerobe *C. pasteurianum*—an organism that contains an active pyruvate synthase system (7).

### Table V

| Treatment | Total 14C-bicarbonate fixed | 14C-Phenylpyruvate formed | 14C-Phenylalanine formed | cpm |
|-----------|-----------------------------|---------------------------|-------------------------|-----|
| Control   | 14,300                      | 9,200                     | 4,100                   | 12,100 |
| Glutamate added | 12,100                      | 2,100                     | 8,700                   | 330  |
| Glutamine added | 12,400                      | 3,300                     | 8,200                   | 380  |

### Table VI

| Treatment | 14C-Bicarbonate incorporated into phenylpyruvate | cpm |
|-----------|-----------------------------------------------|-----|
| Complete  |                                               | 39,900 |
| Phenylpyruvate omitted |                          | 210  |
| CoA omitted |                                         | 2,300 |
| Thiamine pyrophosphate omitted |                  | 2,400 |
| Enzyme preparation omitted |                          | 160  |
| Complete, but CoA replaced by Mercaptoethanol |                  | 3,070 |
| Dithiothreitol |                                         | 2,420 |
| Cysteine |                                         | 3,900 |
| Glutathione |                                         | 5,150 |
| NADH |                                         | 5,020 |
| NADPH |                                         | 1,380 |
| Sodium dithionite |                                        | 13,900 |

### REFERENCES

1. Tagawa, K., and Arnon, D. I., *Nature*, 195, 537 (1962).
2. Tagawa, K., and Arnon, D. I., *Biochim. Biophys. Acta*, 153, 602 (1968).
3. Buchanan, B. B., Bachofen, R., and Arnon, D. I., *Proc. Nat. Acad. Sci. U. S. A.*, 52, 859 (1964).
4. Buchanan, B. B., Evans, M. C. W., and Arnon, D. I., *Arch. Mikrobiol.*, 59, 32 (1967).
5. Evans, M. C. W., and Buchanan, B. B., Proc. Nat. Acad. Sci. U. S. A., 53, 1420 (1965).
6. Evans, M. C. W., Biochem. Biophys. Res. Commun., 33, 146 (1968).
7. Bachofen, K., Buchanan, B. B., and Arnon, D. I., Proc. Nat. Acad. Sci. U. S. A., 61, 660 (1964).
8. Raeburn, S., and Rabinowitz, J. C., Biochem. Biophys. Res. Commun., 18, 303 (1965).
9. Raeburn, S., and Rabinowitz, J. C., in A. San Pietro (Editor), Non-heme iron proteins: role in energy conversion, Antioch Press, Yellow Springs, Ohio, 1965, p. 189.
10. Andrew, I. G., and Morris, J. G., Biochim. Biophys. Acta, 97, 176 (1965).
11. Stern, J. R., in A. San Pietro (Editor), Non-heme iron proteins: role in energy conversion, Antioch Press, Yellow Springs, Ohio, 1965, p. 199.
12. Iker, E., and Bachofen, R., Arch. Mikrobiol., 54, 1 (1966).
13. Buchanan, B. B., and Evans, M. C. W., Proc. Nat. Acad. Sci. U. S. A., 54, 1212 (1965).
14. Allison, M. J., and Robinson, I. M., J. Bacteriol., 104, 50 (1970).
15. Buchanan, B. B., J. Biol. Chem., 244, 4218 (1969).
16. Evans, M. C. W., Buchanan, B. B., and Arnon, D. I., Proc. Nat. Acad. Sci. U. S. A., 55, 928 (1966).
17. Buchanan, B. B., and Arnon, D. I., in J. M. Lowenstein (Editor), Methods in enzymology, Vol. 13, Academic Press, New York, 1969, p. 170.
18. Ioka, M., Trebst, A. V., Ogawa, S., and Arnon, D. I., Nature, 186, 753 (1960).
19. Putzer, R. C., Smillie, E. C., Sisler, E. C., and Kornberg, H. J., J. Biol. Chem., 234, 2130 (1961).
20. Hoare, D. S., Biochem. J., 87, 284 (1963).
21. Arnon, D. I., Das, V. S. R., and Anderson, J. D., in Japanese Society of Plant Physiologists (Editor), Studies on microalgae and photosynthetic bacteria, Univ. of Tokyo Press, Tokyo, 1963, p. 528.
22. Cardin, R., and Osborne, J. G., Biochem. J., 120, 399 (1970).
23. Allison, M. J., and Robinson, I. M., J. Bacteriol., 83, 1209 (1967).
24. Allison, M. J., Biochem. Biophys. Res. Commun., 18, 30 (1965).
25. Beisenherz, G., Bolzze, H. J., Bueche, T., Croz, R., Garad, K. H., Meyer-Arendt, E., and Pfleiderer, G., Z. Naturforsch., 8b, 557 (1953).
26. Morein, L. E., Biochim. Biophys. Acta, 81, 71 (1964).
27. Bachofen, R., and Arnon, D. I., Biochim. Biophys. Acta, 120, 259 (1966).
28. Buchanan, B. B., Matsubara, H., and Evans, M. C. W., Biochim. Biophys. Acta, 189, 46 (1969).
29. Yoch, D. C., Benemann, J. R., Valentine, R. C., and Arnon, D. I., Proc. Nat. Acad. Sci. U. S. A., 64, 1404 (1969).
30. Lowry, O. H., Rosebrugh, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
31. Whatley, F. R., and Arnon, D. I., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 6, Academic Press, New York, 1963, p. 308.
32. Simon, E. J., and Stemin, D., J. Amer. Chem. Soc., 75, 2559 (1953).
33. Stadtman, E. R., J. Biol. Chem., 196, 555 (1952).
34. Autenrieth, W., and Thomas, G., Chem. Ber., 57, 423 (1924).
35. Koch, J., and Stokstad, E. L., Biochem. Biophys. Res. Commun., 23, 586 (1966).
36. Altmann, S. M., Crook, E. M., and Datta, S. P., Biochem. J., 49, 185 (1951).
37. Snyder, F., Anal. Biochem., 9, 183 (1964).
38. Buchanan, B. B., Evans, M. C. W., and Arnon, D. I., in A. San Pietro (Editor), Non-heme iron proteins: role in energy conversion, Antioch Press, Yellow Springs, Ohio, 1965, p. 175.
Ferredoxin-dependent Phenylpyruvate Synthesis by Cell-free Preparations of Photosynthetic Bacteria
Ulrich Gehring and Daniel I. Arnon

J. Biol. Chem. 1971, 246:4518-4522.

Access the most updated version of this article at http://www.jbc.org/content/246/14/4518

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/14/4518.full.html#ref-list-1