ADDED IN PROOF

Gehring (1984) has observed α-H and β-H exchange in mitochondrial aspartate aminotransferase.

ACKNOWLEDGMENTS

We are grateful to Dr. R. A. Porter for the design of the NMR data acquisition, to Lynn Abell for obtaining the NMR spectra, to Dr. Joseph Holtum, Chrissl Roeske, and Edward Geer for help with the vacuum line and the mass spectrometer, and to Dr. W. W. Cleland, Lynn Abell, Chrissl Roeske, and Jeff Hermes for helpful discussions.

Registry No. H_2, 1333-74-0; ^{13}C, 14762-74-4; aspartate β-decarboxylase, 9024-57-1; pyridoxal 5′-phosphate, 54-47-7.

REFERENCES

Albery, W. J., & Knowles, J. R. (1976a) *Biochemistry 15*, 5627–5631.
Albery, W. J., & Knowles, J. R. (1976b) *Biochemistry 15*, 5631–5640.
Babu, U. M., & Johnston, R. B. (1974) *Biochem. Biophys. Res. Commun. 58*, 460–466.
Babu, U. M., & Johnston, R. B. (1976) *Biochemistry 15*, 5671–5678.
Becker, E. D. (1980) *High Resolution NMR*, pp 149–167, Academic Press, New York.
Chang, C.-C., Laghai, A., O'Leary, M. H., & Floss, H. G. (1982) *J. Biol. Chem. 257*, 3564–3569.
Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.
Cleland, W. W. (1975) *Biochemistry 14*, 3220–3224.
Dunn, G. E., & Buccini, J. (1968) *Can. J. Chem. 46*, 563–566.
Gehring, H. (1984) *Biochemistry 23*, 6335–6340.
Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188–190.
Golichowski, A., Haruff, R. C., & Jenkins, W. T. (1977) *Arch. Biochem. Biophys. 178*, 459–467.
Huang, H. H., & Long, F. A. (1969) *J. Am. Chem. Soc. 91*, 2872–2875.
Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105–111.
Metzler, C. M., Cahill, A., & Metzler, D. E. (1980) *J. Am. Chem. Soc. 102*, 6075–6082.
Novogrodsky, A., & Meister, A. (1964) *J. Biol. Chem. 239*, 879–888.
O'Leary, M. H. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Ed.) pp 285–316, Plenum Press, New York.
O'Leary, M. H. (1980) *Methods Enzymol. 64B*, 83–104.
O'Leary, M. H., & Marlier, J. F. (1979) *J. Am. Chem. Soc. 101*, 3300–3306.
O'Leary, M. H., & Piazza, G. (1981) *Biochemistry 20*, 2743–2748.
O'Leary, M. H., Richards, D. T., & Hendrickson, D. W. (1970) *J. Am. Chem. Soc. 92*, 4435–4440.
O'Leary, M. H., Yamada, Y., & Yapp, C. J. (1981) *Biochemistry 20*, 1476–1481.
Ray, W. J., Jr. (1983) *Biochemistry 22*, 4625–4637.
Seltzer, S., Hamilton, G. A., & Westheimer, F. H. (1959) *J. Am. Chem. Soc. 81*, 4018–4024.
Shibatani, T., Kakimoto, T., Kato, J., Nishimura, N., & Chibata, I. (1974) *Hakko Kogaku Zasshi 52*, 886–898.
Sizer, I. W., & Jenkins, W. T. (1962) *Methods Enzymol 5*, 677–684.
Tate, S. S., & Meister, A. (1969) *Biochemistry 8*, 1660–1668.
Tate, S. S., Novogrodsky, A., Soda, K., Miles, E. W., & Meister, A. (1970) *Methods Enzymol. 17A*, 681–688.
Tong, J. Y., & Yankwich, P. E. (1957) *J. Phys. Chem. 61*, 540–543.
Ubreit, W. W., Burris, R. H., & Stauffer, J. F. (1972) *Manometric and Biochemical Techniques*, 5th Ed., p. 100, Burgess, Minneapolis, MN.
Weng, S. H., & Leussing, D. L. (1983) *J. Am. Chem. Soc. 105*, 4082–4090.

Carbon Isotope Effect on Carboxylation of Ribulose Bisphosphate Catalyzed by Ribulosebisphosphate Carboxylase from *Rhodospirillum rubrum*†

C. A. Roeske and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received August 30, 1984

ABSTRACT: The carbon isotope effect at CO_2 has been measured in the carboxylation of ribulose 1,5-bisphosphate by the ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*. The isotope effect is obtained by comparing the isotopic composition of carbon 1 of the 3-phosphoglyceric acid formed in the reaction with that of the carbon dioxide source. A correction is made for carbon 1 of 3-phosphoglyceric acid which arises from carbon 3 of the starting ribulose bisphosphate. The isotope effect is $k^{12}/k^{13} = 1.0178 \pm 0.0008$ at 25 °C, pH 7.8. This value is smaller than the corresponding value for the spinach enzyme. It appears that substrate addition with the *R. rubrum* enzyme is principally ordered, with ribulose bisphosphate binding first, whereas substrate addition is random with the spinach enzyme. The carboxylation step is partially rate limiting with both enzymes.

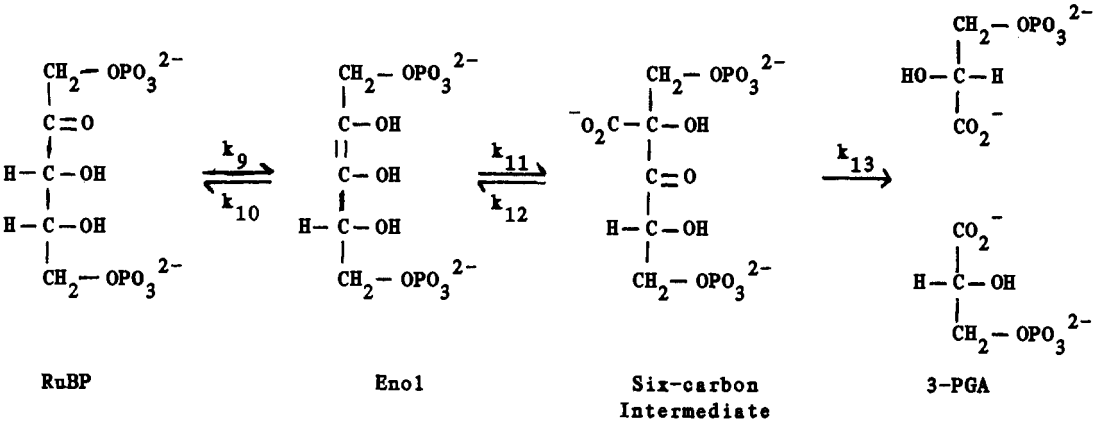
Ribulose bisphosphate (RuBP)[1] carboxylase, the key carbon-fixing enzyme in plants, has been isolated and characterized from a number of green plants and photosynthetic bacteria (Akazawa, 1979). The enzyme from all sources studied to date catalyzes both carboxylation and oxygenation

† This work was supported by Grants PCM-7922697 and PCM-8216597 from the National Science Foundation.
* Address correspondence to this author at the Department of Chemistry, University of Wisconsin—Madison.

[1] Abbreviations: DTT, dithiothreitol; 3-PGA, 3-phosphoglyceric acid; RuBP, ribulose bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

0006-2960/85/0424-1603$01.50/0

Scheme I



of RuBP. The carboxylation reaction is shown in Scheme I. Evidence for the mechanism as depicted here has been reviewed (Miziorko & Lorimer, 1983). The enzyme from spinach and other higher plants has eight large and eight small subunits (Nishimura & Akazawa, 1973), whereas that from the photosynthetic bacterium *Rhodospirillum rubrum* has two large subunits and lacks the small subunit (Tabita & McFadden, 1974).

Although similar in many respects, the enzymes from spinach and *R. rubrum* exhibit different kinetic properties. Perhaps the most physiologically significant of these is the 15-fold difference in the Michaelis constants for CO_2 [20 μM for the spinach enzyme (Badger & Andrews, 1974) vs. 300 μM for the *R. rubrum* enzyme (Christeller & Laing, 1978)]. Both enzymes have unusually low turnover numbers [3 and 6 μmol of CO_2 s^{-1} (active site)$^{-1}$ for spinach and *R. rubrum* enzyme, respectively]. The reason for such inefficient catalysis is unknown. To compensate for it, plants have high concentrations of the enzyme (Kung, 1976).

Isotope effects on enzymic reactions can be analyzed in terms of the intrinsic isotope effect, which is the isotope effect on the single isotope-sensitive bond-breaking or -making step, and the forward and reverse commitment factors (Northrop, 1981). Commitment factors reflect how readily bound species return to substrate or proceed on to product. For example, if a small fraction of substrate is released from E·S compared to the amount converted to product, then the forward commitment is high, and the observed isotope effect is smaller than the intrinsic isotope effect.

The general form of the equation describing the isotope effect on V_{max}/K_m is

$$\frac{V/K}{(V/K)^*} = \frac{k/k^* + C_f + C_r K_{eq}/K_{eq}^*}{1.0 + C_f + C_r}$$

where the asterisk denotes the heavier isotope, k/k^* is the intrinsic isotope effect, C_f and C_r are the forward and reverse commitment factors, respectively, and K_{eq}/K_{eq}^* is the isotope effect on the equilibrium constant. As can be seen from this equation, an increase in the forward or reverse commitment factor decreases the observed isotope effect. The commitment factors can be written as a function of the ratios of rate constants for a particular kinetic mechanism. A change in a commitment factor can therefore be analyzed in terms of the relative rates of steps in the mechanism.

Deuterium and carbon isotope effects for RuBP carboxylase from spinach have been reported. The relatively large isotope effect at CO_2 [$(V/K)^{12}/(V/K)^{13}$ = 1.029; Roeske & O'Leary, 1984] and the intermediate-sized deuterium isotope effect [$(V/K)^H/(V/K)^D$ = 2.5; Schloss, 1983] indicate that both carboxylation and enolization are partially rate determining. The fact that neither isotope effect varies over a range of substrate concentrations is most consistent with a random mechanism, although an ordered addition with CO_2 binding first cannot be completely ruled out (Roeske & O'Leary, 1984).

One explanation for the low turnover number of RuBP carboxylase would be that the enzyme is a poor catalyst because the carboxylation step is inefficient and is quite slow relative to other steps in the reaction. However, the above isotope effects indicate that at least in the case of the spinach enzyme, this does not occur.

Deuteration of RuBP at carbon 3 decreases the isotope effect at CO_2 to 1.021 (Roeske & O'Leary, 1984), implying that CO_2 binds to the enzyme prior to the enolization of RuBP. This rules out the possibility that the enol of RuBP at the active site is formed prior to the binding of CO_2 to the enzyme.

The carbon and deuterium isotope effects for the spinach enzyme have also been used to estimate partition factors for the reaction. The kinetic model for the spinach enzyme is a random mechanism, in which RuBP and CO_2 are released from the ternary complex at similar rates. The carboxylation step is largely or totally irreversible (J. Schloss, personal communication). The observed isotope effects indicate that carboxylation must be 3–5 times slower than reprotonation of the enol (k_{10}/k_{11} = 3–5) (Roeske & O'Leary, 1984) (see Scheme I). One would expect the enzyme from *R. rubrum* to have a different isotope effect at CO_2 than the spinach enzyme because of the large difference in the Michaelis constants for CO_2. Unfortunately, the direction of the change cannot be predicted because of the mathematical complexity of the relationship of K_m to the commitment factors. By comparing the isotope effects at CO_2 with enzymes from two sources, we can compare kinetic mechanisms and relative rates of various steps for enzymes from the two sources.

Experimental Procedures

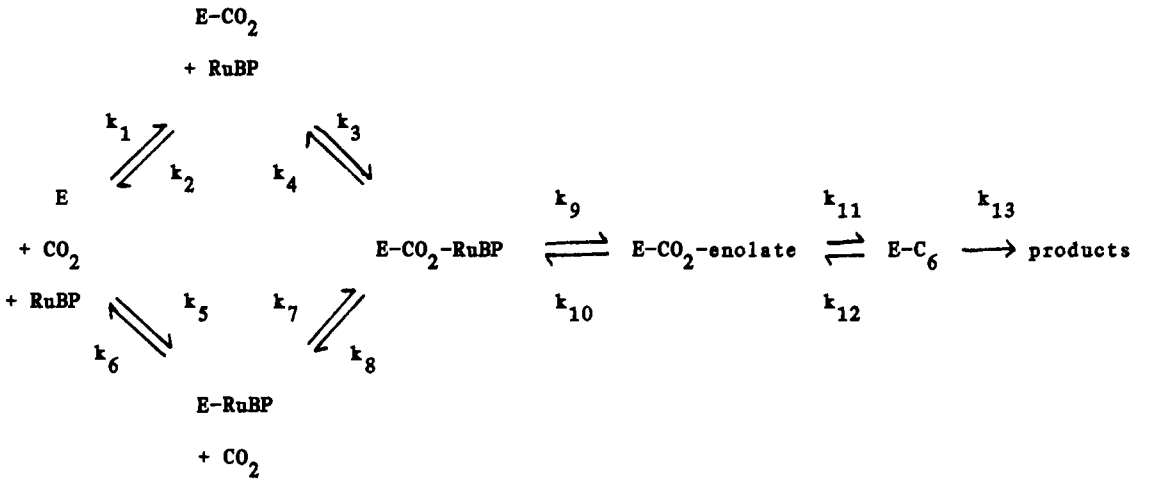
RuBP carboxylase from *R. rubrum* was a gift from Dr. John Schloss. RuBP·2Ba^{2+} was a gift from Dr. George Whitesides. Methods were as previously described (Roeske & O'Leary, 1984).

δ values are defined as follows (Craig, 1957):

$$\delta(^{13}C)\ (‰) = \left(\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{standard}} - 1 \right) 10^3$$

$\delta(^{13}C)$ of each sample was determined on a Finnigan MAT Delta E isotope ratio mass spectrometer. The equations used in determining the isotope effect are as described previously (Roeske & O'Leary, 1984). $\delta(CO_2(aq))$ at pH 7.8 was derived

Scheme II



from $\delta(HCO_3^- + CO_2(aq))$ by using an equation similar to that used at pH 8 (Roeske & O'Leary, 1984). The δ value of carbon 1 of the 3-PGA of interest was calculated from the δ value of carbon 1 of both molecules of 3-PGA and corrected for the δ value of carbon 1 of the lower molecule of 3-PGA (Scheme I), which is carbon 3 of RuBP. δ(C-3, RuBP) was independently determined to be -23.2 ± 0.5‰ by running the oxygenase reaction with enzyme from *R. rubrum* in the presence of Co^{2+} (Roeske & O'Leary, 1984).

Results

The kinetic isotope effect at CO_2 for the reaction catalyzed by RuBP carboxylase from *Rhodospirillum rubrum* was determined by comparing the $\delta(^{13}C)$ values of the initial $CO_2(aq)$ pool and carbon 1 of the upper molecule of 3-PGA (resulting from carbon 1 and carbon 2 of RuBP; see Scheme I). The isotope effect at CO_2 with enzyme from *R. rubrum* is 1.0178 ± 0.0008. The results are summarized in Table I.

Table I: Isotope Effect at CO_2 in the Carboxylation of Ribulose Bisphosphate Catalyzed by RuBP Carboxylase from *Rhodospirillum rubrum* at pH 7.8, 25 °C[a]

reaction	$\delta(CO_2$ (aq))[b]	δ(C-1, 3-PGA)	fraction of CO_2 pool used	k^{12}/k^{13}[c]
1	−15.1	−27.6	0.020	1.0176
2	−14.6	−28.1	0.020	1.0191
3	−14.4	−26.8	0.021	1.0167
4	−14.8	−27.5	0.020	1.0178
5	−14.6	−27.4	0.021	0.0178
6	−14.7	−27.6	0.021	1.0181
				av: 1.0178 ± 0.0008

[a] Reactions were started by the addition of 3 mL of 8.0 mM RuBP to 9-mL solutions containing 4 units of activated RuBP carboxylase (Lorimer et al., 1977), carbonic anhydrase, and the following compounds with final concentrations as given: 70 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM Bicine at pH 7.8. [b] Calculated from $\delta(HCO_3^- + CO_2(aq))$ as previously described (Roeske & O'Leary, 1984). [c] Calculated as previously described (Roeske & O'Leary, 1984). δ-(C-3, RuBP) = -23.2 ± 0.5.

Discussion

The isotope effect at CO_2 on the RuBP carboxylase reaction has been measured a number of times (Park & Epstein, 1960; Deleens et al., 1974; Whelan et al., 1973; Wong et al., 1979; Christeller et al., 1976; Estep et al., 1978; Winkler et al., 1982), with reported values ranging from 1.01 to 1.07. Since these values were obtained by combustion, a likely source of error is the presence of impurities in the RuBP and 3-PGA. This error is magnified by a factor of 5 as a result of correcting for carbon atoms in 3-PGA that come from RuBP. The most recent values obtained with enzyme from higher plants by combustion are 1.028 (Christeller et al., 1976) and 1.029 (Wong et al., 1979). Analysis of the change in the isotopic composition of the starting CO_2 gave an isotope effect of 1.026 (Winkler et al., 1982). All of these more recent values agree with the value obtained with the spinach enzyme by our method of product analysis (Roeske & O'Leary, 1984).

In contrast to the number of experiments done with enzyme from higher plants, the isotope effect with the *R. rubrum* enzyme has been determined only once (Estep et al., 1978), and the value obtained (1.034) does not agree with the value obtained in the present investigation.

For the spinach enzyme, the isotope effects indicate that substrate binding/dissociation, enolization, and carboxylation steps all contribute to the observed rate. The smaller isotope effect obtained with *R. rubrum* enzyme is surprising. If the bacterial enzyme is less highly evolved, one might expect either enolization (k_9) or carboxylation (k_{11}) to be more rate determining than with the spinach enzyme (see Scheme II). Everything else being equal, either the ^2H or the ^{13}C isotope effect would then be larger than with the *R. rubrum* enzyme. However, this is not the case. The carbon isotope effect with the *R. rubrum* enzyme reported here, 1.0178, is substantially smaller than the isotope effect observed with the spinach enzyme (1.029 at pH 8.0). Both are smaller than the intrinsic isotope effect of 1.03–1.07 expected in a carboxylation (Dunn, 1977). Thus, we believe that the carboxylation step is partially rate determining in both cases. The deuterium isotope effect (on V_{max}/K_m) is also smaller for the enzyme from *R. rubrum*, being 1.0 at pH 7.8 (Schloss, 1983; Sue & Knowles, 1982) compared to 2.5 at pH 8.0 with the spinach enzyme (Schloss, 1983). Enolization is therefore partially rate determining with the spinach enzyme but not with the enzyme from *R. rubrum*.

The starting point for comparing carbon isotope effects for enzymes from spinach and *R. rubrum* is the assumption that the intrinsic isotope effects are the same for both enzymes. This assumption follows from the fact that both enzymes catalyze the same reaction at approximately the same rate by using essentially the same mechanism. Therefore, it is reasonable to assume that the transition-state structures for the carboxylation step, and therefore the carbon isotope effects on this step, are similar.

The relative rates of steps (partition factors) in the kinetic mechanism for the *R. rubrum* enzyme were estimated in order to compare them to the values calculated for the spinach enzyme (see the introduction). Calculations were done by using equations for the deuterium and carbon isotope effects

of the general form used previously (Roeske & O'Leary, 1984) and a possible range of 1.04–1.07 for the carbon isotope effect. The kinetic mechanism was assumed to have a random addition of CO_2 and RuBP (i.e., no preferred order), like the enzyme from spinach. With the spinach enzyme, carboxylation is irreversible. However, with the *R. rubrum* enzyme, carboxylation is reversible, the partitioning of the six-carbon intermediate favoring product over starting material by 7:3 (Jaworowski & Rose, 1984). This difference will tend to make the isotope effect smaller for the *R. rubrum* enzyme than for the spinach enzyme. However, if the reversible carboxylation were the only difference between the two enzymes, the partitioning for the *R. rubrum* enzyme would have to be about 1:40 instead of the 7:3 which is observed. Thus, the reversibility of the carboxylation of the *R. rubrum* enzyme cannot be the sole difference between the two enzymes. There must also be differences in the relative rates of the various steps prior to carboxylation.

It has been shown that RuBP carboxylase from *R. rubrum* catalyzes the exchange of protons from solvent into carbon 3 of RuBP (Saver & Knowles, 1982). When this is included in the model for calculating the deuterium isotope effect, then the solutions for the partition factors differ in that formation of the enol (k_9) must become more rapid compared to release of either substrate (k_4 or k_8) than when deuterium washout does not occur. In the limit in which proton exchange is rapid compared to carboxylation, all of the enol becomes reprotonated (rather than deuterated) before carboxylation occurs, so that the reaction occurs along an alternate pathway in which [^{1}H]RuBP is used instead of [^{2}H]RuBP (i.e., the reverse commitment for [3-^{2}H]RuBP becomes zero). This is probably not the case, since carboxylation is not rate determining. However, it is not possible to say anything more specific about the extent of ^{2}H washout since it depends on the relative rates of both carboxylation and reprotonation of the enol (k_{11}/k_{10}) and also on the rate of proton exchange with solvent at the active site. In doing the calculations discussed below, the effect of ^{2}H washout was neglected. As a result, enolization is probably more rapid compared to release of substrate than is indicated by the values below.

Although the partition factors calculated for the random model have a wide range of possible values, two useful comparisons can be made with the values obtained for the spinach enzyme. The first is that with both enzymes the carboxylation step is slower than reprotonation of the enol intermediate ($k_{11}/k_{10} < 1$), the ratio being smaller with the spinach enzyme. Second, while the ternary complex reverts to the E·CO_2 and E·RuBP complexes at approximately equal rates with the spinach enzyme, release of CO_2 is significantly faster than release of RuBP with the *R. rubrum* enzyme. Thus, while the mechanism may still be random, the path in which RuBP binds first is preferred, so that the mechanism begins to look ordered, with RuBP binding first. Indeed, unlike the isotope effects with RuBP carboxylase from spinach, those for the *R. rubrum* enzyme can be readily explained by a completely ordered kinetic mechanism in which RuBP binds first. As with the random mechanism, there are too many parameters to allow unique values of the partition factors in the ordered mechanism. However, if partitioning of the six-carbon intermediate is 2–3 times faster forward than back to CO_2 and enol (k_{13}/k_{12} = 2–3), as required by the results of Jaworowski & Rose (1984), then carboxylation of the enol is several times slower than reprotonation, and enolization is several times faster than release of CO_2 from the ternary complex. None of these partition factors appear unreasonable, so that the *R. rubrum* enzyme, unlike the enzyme from spinach, may have an ordered kinetic mechanism.

In theory, one way to distinguish between the ordered and random mechanisms is to look at the variation of the ^{2}H and ^{13}C isotope effects with substrate concentration (Cleland, 1982). Assuming a random mechanism for RuBP carboxylase, the carbon isotope effect should vary with the concentration of RuBP and the deuterium isotope effect with the concentration of CO_2. If the mechanism is ordered with RuBP binding first, then the carbon isotope effect will be constant as the concentration of RuBP is varied, and the deuterium isotope effect on V_{max}/K_m will vary with the concentration of CO_2. Unfortunately, one cannot distinguish between the random and ordered mechanisms described above because in the random mechanism the slow release of RuBP compared to enolization makes the carbon isotope effect insensitive to variations in the concentration of RuBP. The deuterium isotope effect, however, should be more sensitive to the level of CO_2, increasing at lower concentrations.

A number of differences are now known between RuBP carboxylase from spinach and from *R. rubrum* on the basis of results in this paper and others. The enzymes are structurally distinct, the most notable difference being the small subunits in the spinach enzyme which are not present in the enzyme from *R. rubrum* (Tabita & McFadden, 1974). While this structural difference is certainly reflected in the kinetics, the role of the small subunit remains unknown. Two major differences have been found in the kinetic mechanisms. First, the six-carbon intermediate formed by the carboxylation step decarboxylates little, if at all, to CO_2 plus enol on the spinach enzyme (J. Schloss, personal communication), whereas on the *R. rubrum* enzyme, the six-carbon intermediate decarboxylates about 30% of the time (Jaworowski & Rose, 1984). Second, substrate addition to the spinach enzyme appears to be random, whereas that to the bacterial enzyme probably occurs primarily via the pathway in which RuBP binds first.

ACKNOWLEDGMENTS

We thank Prof. George Whitesides for a generous gift of RuBP and Dr. John Schloss for providing the enzyme used in this study and for permission to quote unpublished information.

REFERENCES

Akazawa, T. (1979) *Encycl. Plant Physiol., New Ser. 6*, 208–229.

Badger, M. R., & Andrews, T. J. (1974) *Biochem. Biophys. Res. Commun. 60*, 204–210.

Christeller, J. T., & Laing, W. A. (1978) *Biochem. J. 173*, 467–473.

Christeller, J. T., Laing, W. A., & Troughton, J. H. (1976) *Plant Physiol. 57*, 580–582.

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385–428.

Craig, H. (1957) *Geochim. Cosmochim. Acta 12*, 133–149.

Deleens, E., Lerman, J. C., Nato, A., & Moys, A. (1974) *Proc. Int. Congr. Photosynth., 3rd*, 1267–1276.

Dunn, G. E. (1977) *Isot. Org. Chem. 3*, 1–40.

Estep, M. F., Tabita, F. R., Parker, P. L., & Van Baalen, C. (1978) *Plant Physiol. 61*, 680–687.

Jaworowski, A., & Rose, I. A. (1984) *J. Biol. Chem. 259*, 6783–6788.

Kung, S. D. (1976) *Science (Washington, D.C.) 191*, 429–434.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1977) *Anal. Biochem. 78*, 66–75.

Miziorko, H. M., & Lorimer, G. H. (1983) *Annu. Rev. Biochem. 52*, 507–535.

Nishimura, M., & Akazawa, T. (1973) *Biochem. Biophys. Res. Commun. 54*, 842–848.
Northrop, D. B. (1981) *Annu. Rev. Biochem. 50*, 103–131.
Park, R., & Epstein, S. (1960) *Geochim. Cosmochim. Acta 21*, 110–126.
Roeske, C., & O'Leary, M. H. (1984) *Biochemistry 23*, 6275–6284.
Saver, B. G., & Knowles, J. R. (1982) *Biochemistry 21*, 5398–5403.
Schloss, J. V. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1923.
Sue, J. M., & Knowles, J. R. (1982) *Biochemistry 21*, 5410–5414.
Tabita, F. R., & McFadden, B. A. (1974) *J. Biol. Chem. 249*, 3459–3464.
Whelan, C. T., Sackett, W. M., & Benedict, C. R. (1973) *Plant Physiol. 51*, 1051–1054.
Winkler, F. J., Kexel, H., Kranz, C., & Schmidt, H.-L. (1982) in *Stable Isotopes* (Schmidt, H.-L., Förstel, H., & Heinzinger, K., Eds.) pp 83–89, Elsevier, Amsterdam.
Wong, W. W., Benedict, C. R., & Kohel, R. J. (1979) *Plant Physiol. 63*, 852–856.

Enzymatic and Nonenzymatic Dehydration Reactions of L-Arogenate†

Lolita O. Zamir,‡ Robert Tiberio, Michael Fiske,§ Alan Berry, and Roy A. Jensen*
Center for Somatic-cell Genetics and Biochemistry, State University of New York, Binghamton, New York 13901
Received August 16, 1984

ABSTRACT: L-Arogenate, an immediate precursor of either L-tyrosine, L-phenylalanine, or both in many microorganisms and plants, may undergo two types of dehydration reactions that yield products of increased stability. Under acidic conditions, a facile aromatization attended by loss of the C-4 hydroxyl and the C-1 carboxyl moieties results in quantitative conversion to L-phenylalanine. When aromatization was largely prevented by maintaining pH in the range of 7.5–12, a second dehydration reaction occurred in which the alanyl side chain and the carboxyl group at C-1 formed a lactam ring to yield spiro-arogenate. The latter reaction occurs at 100 °C, roughly 50% conversion being obtained in 2 h. The product formed from L-arogenate was authentic spiro-arogenate, as demonstrated by high-performance liquid chromatography and thin-layer chromatography identification procedures. Further confirmation was obtained by ^1H nuclear magnetic resonance, ultraviolet spectroscopy, and mass spectrometry. Thus far, the conversion of L-arogenate to spiro-arogenate is not known to be enzyme catalyzed. The other dehydratase reaction, however, is catalyzed in nature by an enzyme denoted arogenate dehydratase. An improved assay is described for this in which [^3H]dansyl derivatives of L-arogenate (substrate) and L-phenylalanine (product) are separated by using bidimensional thin-layer chromatography. The radioactive reaction product is then quantitated. This assay was used to study partially purified arogenate dehydratase from *Pseudomonas diminuta*, an organism that depends upon the arogenate pathway for L-phenylalanine biosynthesis. This enzyme possessed a $K_{m,app}$ of 0.63 mM for L-arogenate and was sensitive to inhibition by L-phenylalanine, 50% inhibition being obtained at 70 μM L-phenylalanine. In contrast, arogenate dehydratase isolated from *Pseudomonas aeruginosa*, an organism that possesses both the phenylpyruvate and arogenate routes to L-phenylalanine, was insensitive to inhibition by L-phenylalanine.

L-(8*S*)-Arogenate has taken on steadily increasing significance as an amino acid precursor of L-tyrosine, L-phenylalanine, or both in prokaryotic and eukaryotic microorganisms, as well as in higher plants (Byng et al., 1982). Zamir et al. (1980) showed the structure of L-arogenate to be β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine, the expected transamination product of prephenate. A second cyclohexadienyl structure, isolated by Zamir et al. (1983c) from culture supernatants of *Neurospora crassa*, is the lactam derivative of L-arogenate and was assigned the trivial name spiro-arogenate (spiro[4-hydroxy-2,5-cyclohexadiene-1,γ-L-pyroglutamic acid]).

Under acidic conditions both compounds are aromatized following dehydration to yield L-phenylalanine. At a given pH, the rate of aromatization increases in proportion to elevation of reaction temperature. Acid-catalyzed conversion of spiro-arogenate to L-phenylalanine undoubtedly progresses through a step in which L-arogenate is formed as an intermediate. Figure 1 illustrates a feasible mechanism for acid-catalyzed aromatization of arogenate, a sequence in which the C-4 hydroxyl group is protonated, leading to extrusion of the carboxyl moiety and elimination of water to yield a stable aromatic ring.

We have recently found that at mildly alkaline pH where decarboxylation of L-arogenate is largely prevented, a second type of dehydration reaction, yielding spiro-arogenate, will occur at elevated temperature. The dehydratase reaction that aromatizes L-arogenate is already established (Byng et al.,

† This research was supported by Grant AM-19477 from the National Institutes of Health of the U.S. Public Health Service.
‡ Present address: Department of Chemistry, McGill University, Montreal, Quebec, Canada, and the Centre de Bacteriologie, Universite du Québec, Institut Armand-Frappier 531, Laval-des-Rapides, Laval, Québec, Canada.
§ Present address: Corporate Research Labs, Monsanto Chemical Co., St. Louis, MO 63166.

0006-2960/85/0424-1607$01.50/0