The Carboxylation of Phosphoenolpyruvate and Pyruvate

II. THE ACTIVE SPECIES OF “CO$_2$” UTILIZED BY PHOSPHOENOLPYRUVATE CARBOXYLASE AND PYRUVATE CARBOXYLASE*

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SUMMARY

The active species of “CO$_2$”, i.e., CO$_2$ or HCO$_3^-$, utilized in the pyruvate carboxylase and phosphoenolpyruvate carboxylase reaction have been determined with an assay predicated upon the fact that the hydration of CO$_2$ is a kinetically slow and measurable reaction. The results of these assays in both cases are in reasonable agreement with those expected if HCO$_3^-$ is assumed to be the functional species utilized by both enzymes.

The species of “CO$_2$” (CO$_2$, H$_2$CO$_3$, or HCO$_3^-$) utilized in carboxylation reactions limit both the type of mechanism that may be suggested for the carboxylases involved and the physiological significance attributed to carbonic anhydrase that may be found in the proximity of them. For example the finding (1) that CO$_2$ was actively fixed in the phosphoenolpyruvate carboxykinase reaction necessitated, along with other data (2, 3), modification of the reaction mechanism initially suggested for this enzyme (4, 5). Further the fact (6) that ribulose diphosphate carboxylase uses CO$_2$ and not HCO$_3^-$ required that a new role be sought for the carbonic anhydrase found with the enzyme in the chloroplast (7). Cooper et al. (1, 8) reported a kinetic method for the determination of the species of “CO$_2$” that is initially bound to the enzyme. This work supplemented the previously existing approach of Kaziro et al. (9) using HCO$_3^-$, which determined the species participating in the chemical reaction. Although there was good qualitative agreement between the theoretical and experimental results of these kinetic methods when CO$_2$ was the actively fixed species, the same could not be said when HCO$_3^-$ was shown to be active in the pyruvate carboxylase reaction (compare Fig. 6B and 8 of Reference 1). The criticism of those experiments on pyruvate carboxylase was that all of the samples were taken after the hydration-dehydration reaction had reached equilibrium, i.e., all of the points appear on the linear portion of the curves. These results were obtained with the cold labile, avian pyruvate carboxylase which required that the analysis be performed at 25° instead of the usual 10°. At the higher temperature the hydration-dehydration equilibrium was presumably attained before the first samples were taken.

Waygood, Mache, and Tan (10) have presented kinetic data demonstrating that CO$_2$ is the active species utilized by P-enolpyruvate carboxylase. This report is at variance with the work of Lane's group (4), who, by means of isotopic techniques, obtained data supporting HCO$_3^-$ as the actively fixed species.

In the present studies these two problems have been reinvestigated. The yeast pyruvate carboxylase, which is not subject to cold lability, and thus permits the analysis to be executed at 10° was employed in place of the avian enzyme. The data obtained with the yeast enzyme agree with both the conclusions of the earlier experiments and with the theoretically expected results if HCO$_3^-$ is assumed to be active. The present results also support Lane's contention that HCO$_3^-$ is the actively fixed species of “CO$_2$” in the P-enolpyruvate carboxylase reaction.

METHODS

Avian pyruvate carboxylase was the generous gift of Dr. M. F. Utter. P-enolpyruvate carboxylase was obtained from germinating peanuts and purified by the procedures of Maruyama et al. (4) through the DEAE-chromatographic step of their scheme. P-enolpyruvate carboxylase from corn shoots was prepared in the following manner. Corn shoots (12 to 14 days old) were harvested, suspended in a buffer containing 0.2 M Tris, pH 7.8, 1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol, and homogenized in a Waring Blender. The crude pulp was filtered through cheesecloth, followed by centrifugation of the filterate for 15 min at 15,000 rpm in a Sorvall RC-2B refrigerated centrifuge. The soluble supernatant was then brought to 45% saturation with solid ammonium sulfate and the pellet discarded. Additional ammonium sulfate was added to 60% saturation. Following collection of the precipitated protein by centrifugation, it was dissolved in 0.1 M Tris buffer, pH 7.5 (similar to that described above). This preparation was used without further purification. The analysis of the active species fixed by these carboxylases was performed as described earlier (1).
RESULTS AND DISCUSSION

The method (1, 8) is based upon Roughton’s observation that
\[ \text{H}_2\text{O} + \text{CO}_2 = \text{H}_2\text{CO}_3 = \text{H}^+ + \text{HCO}_3^- \]
requires more than 60 s to attain equilibrium at temperatures
below 15° when the initial reactants are CO2 and H2O. With the
equations developed earlier (8) it is possible to calculate the
progress of product formation (being monitored either spec-
tronomically or radiochemically) as a function of time. These
curves are shown in Fig. 1A for the situation where HCO3- is
assumed to be active and in Fig. 1B for that where CO2 is
assumed to be active.

If CO2 is assumed to be active and is initially added to the
reaction (Fig. 1A, CO2 line), there is a high rate of CO2 fixation
which decays toward a steady state rate after several minutes.
This high rate of synthesis is not observed, however, in the
presence of carbonic anhydrase. If, on the other hand, HCO3- is
assumed to be fixed, then there is a pronounced lag when CO2 is
initially added. Addition of CO2 and carbonic anhydrase
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initially added. Addition of CO2 and carbonic anhydrase
together results in the disappearance of the lag, yielding a rate similar to that seen when HCO$_3^-$ is initially present in the reaction mixture. It should be emphasized that, in distinction to the earlier work (Figs. 2 and 6 of Reference 1), the curves obtained upon addition of CO$_2$ or HCO$_3^-$ to the reaction mixture do not become parallel over the time period that the analysis is conducted. This difference is the result of conducting the experiments at a higher pH, 8.0. This higher pH requires that a larger percentage of the CO$_2$ be converted to HCO$_3^-$ before equilibrium is attained; the parallel portions of the curves in Figs. 2 and 6 of Reference 1 are the manifestation of having attained equilibrium. Since the rate of the hydration-dehydration reactions is a constant with respect to pH, the higher the pH, the greater will be the time required to reach equilibrium. A more detailed account of the theoretical implications of the various physical parameters upon this analysis have appeared elsewhere (8).

Yeast Pyruvate Carboxylase—With the use of pyruvate carboxylase from yeast the spectrophotometric analysis was performed at 10°. As shown in Fig. 2, when HCO$_3^-$ was the initially presented species there was a high and nearly linear rate of fixation. When CO$_2$ was initially present, however, there was a pronounced lag which was obliterated by the addition of CO$_2$ in the presence of excess carbonic anhydrase (filled squares). It is noteworthy that all of these data were collected during the first minute after the reaction was initiated. Results identical with these were obtained over a longer time period when the radiochemical assay was employed (Fig. 3). In both of these experiments the majority of the data were obtained before attainment of the hydration-dehydration equilibrium and hence are not subject to the criticisms of the earlier data (Fig. 9 of Reference 1). The conclusions reached in that previous work, however, are confirmed by the present results.

P-enolpyruvate Carboxylase—Recently Waygood and co-workers (10) have presented data, by means of the analysis described above, which indicate that CO$_2$ is the active species of “CO$_2$” utilized in the P-enolpyruvate carboxylase reaction. Since these data were in conflict with those of Maruyama et al. (4), the kinetic experiments of Waygood were repeated with the more sensitive radiochemical assay described earlier (1). As shown in Fig. 4 there was a high rate of fixation when HCO$_3^-$ was initially present and a much lower rate when $^{14}$CO$_2$ was initially presented as the labeled substrate. The inclusion of carbonic anhydrase increased the observed rate of fixation in a reaction mixture containing CO$_2$ as the initially labeled species. These data are consistent with those expected if HCO$_3^-$ is assumed to be the actively fixed species. Some departure from the theoretically expected results, however, can be observed in these data. The rate of fixation in the presence of carbonic anhydrase and initially added $^{14}$CO$_2$ should approach that attained when HCO$_3^-$ was initially added (Fig. 1B). The actual rate observed was only about 50% of that expected. The reason for this abnormality is not known at the present time, but increasing the carbonic anhydrase concentration did not appreciably improve the situation.

The above data were obtained with an enzyme preparation partially purified from peanut cotyledons (the same source as that used by Lane). As shown in Fig. 5, the same type of results was obtained with P-enolpyruvate carboxylase from maize seedlings (the same source as that used by Waygood). These data are somewhat compromised, however, by the presence of carbonic anhydrase in the enzyme preparation. This contamination results in a higher rate of fixation when $^{14}$CO$_2$ was the initially labeled species than would have otherwise been expected. These experiments support the results of Lane and his co-workers that HCO$_3^-$ and not CO$_2$ is actively fixed by P-enolpyruvate carboxylase.

The approach used in the present studies and those of Waygood is the same. At present there is no explanation for the variance observed between the data obtained in the two laboratories, although the tracer techniques have been found, in our hands, to be somewhat less subject to technical difficulties than the spectrophotometric assays. In addition to the isotopic labeling data of Lane, there is one piece of circumstantial evidence that is consistent with HCO$_3^-$ being the actively utilized species for P-enolpyruvate carboxylase. Filmer and Cooper (8) noted that without exception those carboxylases which utilized CO$_2$ were observed to possess abnormally high (greater than 5 to 10 mM) apparent $K_m$ values for total “CO$_2$” i.e. CO$_2$ + HCO$_3^-$. Where those using HCO$_3^-$ displayed a much lower apparent $K_m$ (below 1 mM) for total “CO$_2$.” It was suggested that the reason behind these findings was that all of the $K_m$ values were determined at alkaline pH where most of the “CO$_2$” is in the form of HCO$_3^-$. Therefore, the amount of CO$_2$ actually present in the reaction mixtures was only a small fraction of the total added. It has been observed (4) that at pH 7.9 the apparent $K_m$ value of P-enolpyruvate carboxylase for total “CO$_2$” was 0.31 mM. A $K_m$ value of this magnitude would be predicted if HCO$_3^-$ is the active species.

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