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MODIFICATION OF RIBULOSE BISPHOSPHATE CARBOXYLASE BY 2,3-BUTADIONE

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SUMMARY: D-ribulose-1,5-bisphosphate carboxylases purified from barley or formate-grown Pseudomonas oxalaticus were inactivated by 2,3-butadione. Pseudo first-order inactivation depended on the presence of borate and was reduced by product 3-phosphoglycerate. The half-times at 30°C and pH 8.3 in the presence of 2 mM 2,3-butadione are 10 and 60 minutes for the enzymes from P. oxalaticus and barley, respectively. Saturation kinetics and arginine modification were demonstrated for the enzyme from P. oxalaticus.

INTRODUCTION: Numerous enzymes that bind anionic substrates or inhibitors contain arginyl residues at the binding sites and share a borate-dependent sensitivity to phenylglyoxal or 2,3-butadione, which, in cases examined, is not evident in the presence of substrates or inhibitors. For example, borate-dependent inactivation of nine of ten glycolytic enzymes by 2,3-butadione has been reported (1). We now describe some characteristics of the borate-dependent inactivation of two different D-ribulose-1,5-bisphosphate (RuBP) carboxylases (EC. 4.1.1.39) by this reagent.

MATERIALS AND METHODS: Ribulose bisphosphate carboxylase from formate-grown Pseudomonas oxalaticus (2) was purified 30-fold from cell-free extracts by precipitation at 25-40% saturation with respect to $(NH_4)_2SO_4$ (pH 7.8), sedimentation into a continuous sucrose gradient (3), and batchwise elution from a DEAE-cellulose column at a concentration of 0.10 M NaCl. The gel electrophoretically homogeneous enzyme and had a final specific activity of 1.6 μmoles RuBP-dependent CO_2 fixed/min/mg protein (4).

Ribulose bisphosphate carboxylase from barley (Hordeum vulgare) was purified (final specific activity: 0.7) by A. Saluja by precipitation at 25-50% saturation with respect to $(NH_4)_2SO_4$ (pH 8), sedimentation into a sucrose density gradient (3), and reprecipitation with $(NH_4)_2SO_4$.

In all studies, activated enzyme which had been preincubated in HEMMB buffer, pH 8.0 (50 mM HEPES-Na^+, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 mM $NaHCO_3$), was mixed with an equal volume of water solution containing the reagents (specified at final concentrations). Both the enzyme and the reagent solution had been equilibrated to 30°C unless otherwise indicated.

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For assay (5), 20 µl of the resultant mixture containing the P. oxalaticus enzyme was added to 230 µl (30°C) of a solution, pH 8.0 (25°C), of 5.0 µmoles $NaH^{14}CO_3$, 0.5 µmoles EDTA, 4.0 µmoles $MgCl_2$ and 0.2 µmoles RuBP (tetrasodium; Sigma) and 15.5 µmoles Tris. For the barley enzyme, 50 µl of the resultant mixture was added to 200 µl containing the constituents specified above. Reactions were conducted for 5 minutes and terminated with 100 µl of 60% cold trichloroacetic acid. After removal of 200 µl of sample, excess $^{14}CO_2$ was liberated at 70-85°C for 45 minutes prior to counting (5).

Arginine analyses were performed in duplicate on the short column of a Beckman 121 Automatic Amino Acid Analyzer after hydrolysis of samples for 24 hours at 110°C in 6.0 N HCl.

RESULTS: When RuBP carboxylase from P. oxalaticus was incubated in the presence of 50 mM borate and 2 mM 2,3-butadione (Aldrich; redistilled every 14 days and stored desiccated at -20°C), a marked decrease in enzymatic activity was observed with respect to the control (in HEMMB plus borate). The inactivation appears to be pseudo-first order with a $t_{1/2}$ of 10 minutes in the presence of 2 mM 2,3-butadione and 50 mM borate (Fig. 1a). In the absence of borate or in the presence of 10 mM 3-phosphoglycerate (Sigma; disodium salt) plus 50 mM borate, $t_{1/2}$ was greater than 60 minutes. The possibility that 3-phosphoglycerate, the product of the reaction, had protected by reacting with one or one-half equivalent of borate was ruled out by performing the inactivation with 40 mM borate and 2 mM 2,3-butadione. The rate of inactivation under these conditions was identical to that occurring in the presence of 50 mM borate (Fig. 1a). Clearly, then, 3-phosphoglycerate protects against inactivation. Even at a concentration of 20 mM 2,3-butadione (with 50 mM borate), the residual activity after 10 minutes in the presence of 10 mM 3-phosphoglycerate was 7.5-fold higher than that in its absence (8%). The activity of enzyme which had been 87%-inactivated by 2.0 mM 2,3-butadione could be doubled by overnight dialysis at 4°C against HEMMB followed by immediate assay. Failure to achieve full reversal of inactivation has been experienced in other labs (for example, ref. 6).

Similar results were obtained with 25 mM Tris-Cl (pH 8.0, 25°C) instead of HEPES. However, much higher concentrations of 2,3-butadione were required to achieve comparable rates of inactivation in Tris buffer. The $t_{1/2}$ in the

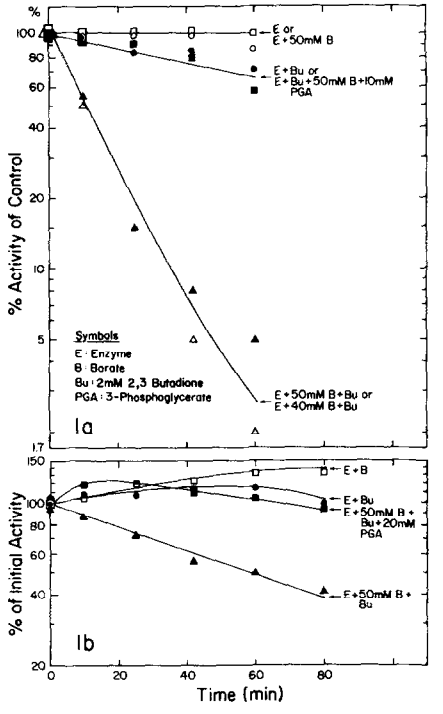

Figure 1a. 100 µg of P. oxalaticus RuBP carboxylase was mixed with an equal volume of an aqueous solution with the following constituents: o—o, Enzyme (E) alone; □—□, E + 50 mM borate (pH 8.3); ■—■, E + 50 mM borate (pH 8.3) + 2.0 mM 2,3-butadione + 10 mM 3-phosphoglycerate; ●—●, E + 2.0 mM 2,3-butadione; Δ—Δ, E + 40 mM borate (pH 8.3) + 2.0 mM 2,3-butadione; and ▲—▲, E + 50 mM borate (pH 8.3) + 2.0 mM 2,3-butadione. E + borate (□—□) throughout the time course was considered to be 100% activity and other data are compared with this value for each time.

Figure 1b. 160 µg of barley RuBP carboxylase was mixed with an equal volume of an aqueous solution containing the following: □—□, enzyme (E) + 50 mM borate (pH 8.3); ■—■, E + 2.0 mM 2,3-butadione + 50 mM borate (pH 8.3) + 2.0 mM 3-phosphoglycerate; ●—●, E + 2 mM 2,3-butadione; and ▲—▲, E + 50 mM borate (pH 8.3) + 2.0 mM 2,3-butadione.

presence of 20 mM 2,3-butadione and 50 mM borate was 36 minutes. The longer $t_{1/2}$ may have been due to the reaction of Tris with 2,3-butadione.

RuBP carboxylase from barley also underwent borate-dependent inactivation by 2.0 mM 2,3-butadione (Fig. 1b). The inactivation was pseudo-first order and had a $t_{1/2}$ of 60 minutes with respect to the zero-time activity. Unlike the P. oxalaticus enzyme the activity of the barley enzyme increased by 40% in 40 minutes upon incubation at 30°C in HEMMB buffer. This may have been due to

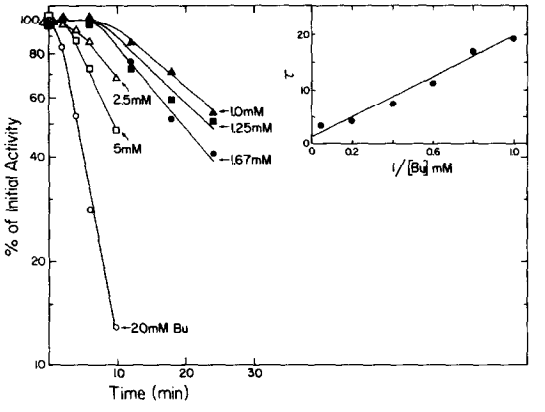

Figure 2. Time-course of inactivation of RuBP carboxylase from P. oxalaticus at varying 2,3-butadione (Bu) concentrations in the presence of 50 mM borate. The incubation was initiated by mixing the enzyme and reagent solution (each at 4°C) as described and instantly warming to 30°C. The inset shows a plot of τ versus 1/[Bu].

thermal activation (7). The inactivation by 2,3-butadione could be prevented by the addition of 20 mM 3-phosphoglycerate (Fig. 1b). In contrast, 10 mM 3-phosphoglycerate provided little protection.

It is noteworthy that with both enzymes, inactivation occurred in the presence of 25 mM HCO_3^- (and 5 mM Mg^{+2}). HCO_3^- is in equilibrium with CO_2, one of the two substrates (8). Thus a combination of the required metal ion and one of two substrates provided little or no protection.

When the P. oxalaticus enzyme was subjected to various concentrations of 2,3-butadione, the inactivation was slower in the initial phase. The length of the lag depended upon the concentration of the reagent (Fig. 2). At the end of this lag, the inactivation became pseudo-first order. If the overall half-time of inactivation (including the lag period) is defined as τ where $\tau = \frac{\ln 2}{k_2} + \frac{\ln 2}{k_2} \frac{K_{inact.}}{[Bu]}$ (9), $K_{inact.}$ = 8 mM and τ_{min} = 3 minutes. However if only the pseudo-first order portion of the line is used (inset, Fig. 2), the values obtained for $K_{inact.}$ and τ_{min} were 17 mM and 1.25 minutes. The lag observed could possibly have been due to equilibration between the borate, 2,3-butadione and enzyme or may have reflected "positive, weak, irreversible-

binding cooperativity" between multiple sites, which could account for the lag reduction at higher inactivator concentrations (10).

When RuBP carboxylase from P. oxalaticus was incubated for one hour with: (a) 50 mM borate, (b) 50 mM borate, 10 mM 3-phosphoglycerate, and 2.0 mM 2,3-butadione or (c) 50 mM borate and 2.0 mM 2,3-butadione, 90%, 65% and 17% of the zero-time activity was recovered, respectively. After exhaustive dialysis of each sample against 50 mM borate, analysis revealed the loss of 38% of the arginine in the presence of 3-phosphoglycerate (cf., a and b) and 60% in its absence (cf., a and c) whereas no loss of histidine or lysine occurred under any conditions. The incremental loss in arginine that occurred in the absence of 3-phosphoglycerate (cf., b and c) corresponds to 8 arginines per 71,400-dalton combination of one large and one small subunit in this 455,000-dalton enzyme (Lawlis and McFadden, unpublished).

DISCUSSION: In this work it has been shown that RuBP carboxylase from a chemolithotroph and a higher plant undergo a pseudo-first order, borate-dependent inactivation by 2,3-butadione. This inactivation can be prevented by 3-phosphoglycerate, the product of the reaction. Similar results have been obtained with the enzyme from the photolithotroph Thiocapsa (Lawlis and McFadden, unpublished). The requirement of a higher concentration of 3-phosphoglycerate to protect the barley enzyme probably reflects a decreased affinity for the product. McFadden has reviewed the data which establish that 3-phosphoglycerate inhibits both bacterial and higher plant enzymes with K_i values in the range of 8-15 mM (11). There are advantages in the use of 3-phosphoglycerate over RuBP as a protective agent. In the use of RuBP substrate turnover may occur and borate would complex with RuBP complicating interpretations. Of deeper significance is the possibility that more than one type of binding site for RuBP exists (11).

In more detailed studies of the inactivation of RuBP carboxylase from P. oxalaticus, saturation kinetics and reversibility upon removal of 2,3-

butadione and borate have been demonstrated. Inactivation with saturation kinetics and protection by the product (but not by other anionic species such as borate, HCO_3^-, Cl^- or EDTA) are strongly suggestive of specificity and imply a modification of the active site. Additional sites of modification occur, however, as evidenced by the incremental modification of 8 arginines per repeating unit of this enzyme in the absence of 3-phosphoglycerate.

It is tempting to suggest that arginine is present at the active site of RuBP carboxylase from three organisms and, presumably, from all autotrophs. In this context, an indication that the large subunits of this enzyme have been strongly conserved in evolution has been discussed (11,12). These subunits harbor catalytic activity or catalytic potential. It is also relevant that striking similarities between the labeling of RuBP carboxylase from spinach and Rhodospirillum rubrum by the active-site reagent 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate have been reported (13) as have modifications by pyridoxal phosphate of a green algal and two bacterial enzymes (14).

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REFERENCES

1. Riordan, J. F., McElvany, K. D., and Borders, C. L. (1977) Science 195, 884-886.
2. Blackmore, M. A., Quayle, J. R., and Walker, I. O. (1968) Biochem. J. 107, 669-704.
3. Tabita, F. R., and McFadden, B. A. (1974) Arch. Microbiol. 99, 231-240.
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-267.
5. McFadden, B. A., Tabita, F. R., and Kuehn, G. D. (1975) Methods of Enzymology 42, 461-472.
6. Borders, C. L., and Riordan, J. F. (1975) Biochemistry 14, 4699-4704.
7. Chollet, R., and Anderson, L. L. (1977) Biochim. Biophys. Acta 482,228-240.
8. Cooper, T. G., Filmer, D., Wishnick, M., and Lane, M. D. (1969) J. Biol. Chem. 244, 1081-1083.
9. Roche, T. E., and McFadden, B. A. (1969) Biochem. Biophys. Res. Comm. 37, 239-246.
10. Ratkitzis, E. T. (1977) J. Theoret. Biol. 67, 49-59.
11. McFadden, B. A. (1973) Bacteriol. Rev. 37, 284-319.
12. McFadden, B. A., and Tabita, F. R. (1974) Biosystems 6, 93-112.
13. Schloss, J. V., and Hartman, F. C. (1977) Biochem. Biophys. Res. Comm. 75, 320-328.
14. Whitman, W., and Tabita, F. R. (1976) Biochem. Biophys. Res. Commun. 71, 1034-1039.