# REVERSIBLE DESENSITIZATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE TO MULTIPLE EFFECTORS BY BUTANEDIONE

Isamu Kameshita, Masanobu Tokushige, Katsura Izui and Hirohiko Katsuki

Department of Chemistry, Faculty of Science,
Kyoto University, Kyoto, Japan

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## SUMMARY

Chemical modification of phosphoenolpyruvate carboxylase [EC 4.1.1.31] of *Escherichia coli* W with 2,3-butanedione, an arginyl residue reagent, results in an inactivation of the enzyme. The inactivation proceeds following pseudo-first order kinetics. DL-Phospholactate, a substrate analog, effectively protects the enzyme from the inactivation. The enzyme modified in the presence of DL-phospholactate or in its absence is completely desensitized to fructose 1,6-bisphosphate and GTP, allosteric activators for the enzyme. At the same time, the sensitivities to acetyl coenzyme A, laurate and L-aspartate are considerably decreased. Resensitization is attained, however, upon removal of excess butanedione and borate by gel filtration, concomitant with the restoration of the catalytic activity.

## INTRODUCTION

Phosphoenolpyruvate carboxylase [EC 4.1.1.31] of the Enterobacteriaceae is known to be one of the characteristic regulatory enzymes whose activity is controlled by multiple allosteric effectors. The enzyme from *Escherichia coli* W is inhibited by L-aspartate (1) and activated by CoASAc (2), Fru-1,6-$P_2$ (3), GTP and fatty acids (or their CoA derivatives) (4).

Chemical modification studies of the enzyme revealed that cysteinyl (5) and histidyl residues (6) are essential for the catalytic function of the enzyme. Furthermore, histidyl residue was found to be essential for the regulatory interaction with Fru-1,6-$P_2$ and L-aspartate (6). In the present communication, we wish to report that the chemical modification of the enzyme with butanedione caused both inactivation and complete desensitization to Fru-1,6-$P_2$ and GTP. The sensitivities were found, however, to be restored when borate was removed. The available evidence suggests that arginyl residues are involved in the catalytic and the regulatory functions of the enzyme.

## MATERIALS AND METHODS

Fru-1,6-$P_2$ and GTP were purchased from Sigma. PEP, CoASAc and P-lac were prepared as described previously (5). All other chemicals were of reagent grade. PEP carboxylase was purified to homogeneity from *E. coli* W

Abbreviations: Fru-1,6-$P_2$, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; CoASAc, acetyl coenzyme A; P-lac, DL-2-phospholactate





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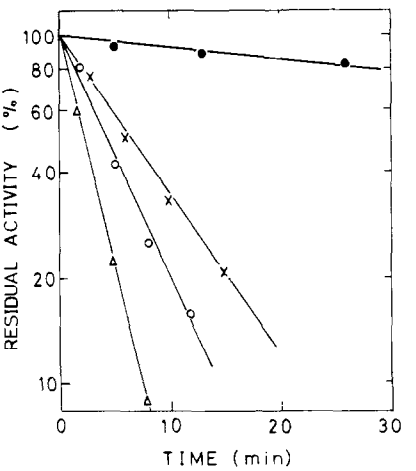


Fig. 1. Semi-log plots of changes in the activity of PEP carboxylase in the presence and absence of P-lac. PEP carboxylase (160 μg protein) was incubated in a total volume of 0.2 ml at 30°C in 50 mM borate buffer (pH 8.5) with the following additions: **X**, 2 mM butanedione; **O**, 5 mM butanedione; **Δ**, 10 mM butanedione; **●**, 0.5 mM CoASAc, 10 mM $MgSO_4$, 50 mM P-lac and 5 mM butanedione. After incubation for the time indicated, 10-μl aliquots were withdrawn and assayed for the residual activity under the standard assay conditions. Although the butanedione modified enzyme gradually restored the activity after added to the assay mixture, it could be neglected, since the assay was carried out in as short as 1 or 2 min.

cells according to the method of Yoshinaga *et al.* (7). The enzyme activity was determined by the measurement of NADH oxidation at 340 nm in a coupled reaction system with malate dehydrogenase [EC 1.1.1.37] at 30°C in a Hitachi 124 recording spectrophotometer equipped with a constant temperature cell housing (7).

Butanedione used for the modification was redistilled and dissolved in 50 mM sodium borate buffer (pH 8.5) prior to use. The enzyme and indicated additions dissolved in the same buffer were preincubated for 10 min at 30°C and butanedione was added to the mixture. The modification reaction was allowed to proceed at the same temperature (total volume of 0.2 ml). At designated time intervals, 10-μl aliquots were withdrawn and assayed for their residual activities to monitor the reaction progress.

Amino acid analysis was carried out according to the method of Riordan (8) using Hitachi KLA-3B amino acid analyzer.

## RESULTS AND DISCUSSION

When PEP carboxylase was incubated with butanedione in 50 mM borate buffer (pH 8.5), a time-dependent inactivation was observed. In Fig. 1 is represented the inactivation on a log scale of the residual activity as a function of time. The time course of inactivation exhibited pseudo-first order kinetics and its rate was dependent on the concentration of

TABLE 1. Alteration of effector sensitivities of PEP carboxylase by modification with butanedione. Modification of the enzyme was carried out as described in Fig. 1, except that the concentration of P-lac was 20 mM. The modified enzyme was desalted as described in the text. The assay mixture contained indicated concentrations of the effectors and the enzyme. The sensitivities were expressed as relative value, taking the enzyme activity in the absence of the effector as a standard.

| Effector* | (mM) | Relative Sensitivity | |
|---|---|---|---|
| | | Native Enzyme | Modified Enzyme** |
| Fru-1,6-$P_2$ | (10.0) | 4.8 | 1.0 |
| GTP | (5.0) | 3.3 | 1.0 |
| CoASAc | (0.2) | 9.6 | 2.6 |
| Laurate | (0.1) | 3.5 | 2.0 |
| L-Aspartate | (5.0) | 0.18 | 0.70 |

* Added in a form of sodium salt except CoASAc.
** The residual activity of the enzyme was 80% of the initial enzyme activity.

butanedione. Virtually no decrease in the enzyme activity occurred during the incubation of the enzyme in the borate buffer under the experimental conditions. When P-lac, a structural analog of PEP (5), was present together with CoASAc and $Mg^{2+}$ ions, a marked protection was observed against the butanedione inactivation. Incubation of the enzyme with 5 mM butanedione resulted in an 80% inactivation in 10 min, whereas in the presence of P-lac, CoASAc and $Mg^{2+}$ ions more than 90% of the initial activity remained. No appreciable protection was observed in the presence of CoASAc and $Mg^{2+}$ ions. These results suggest that the amino acid residues modified with butanedione are located at or near the active site of the enzyme. The modified enzyme was gel-filtered through a small column of Sephadex G-50 (0.6 x 12.0 cm) equilibrated with 50 mM sodium borate buffer (pH 8.5) to remove excess butanedione, and the *Km* value for the substrate and sensitivities to the effectors were examined. The *Km* values for PEP of the native enzyme and the modified enzyme in the presence of P-lac (residual activity, 80%) were found to be 22 and 20 mM, respectively. The results imply that the butanedione modification caused little alteration in the *Km* value for the substrate.

Table 1 shows sensitivities of the native and the modified enzymes to five kinds of effectors—Fru-1,6-$P_2$, GTP, CoASAc, laurate and L-aspartate. The modification caused a complete loss of sensitivities to Fru-1,6-$P_2$ and

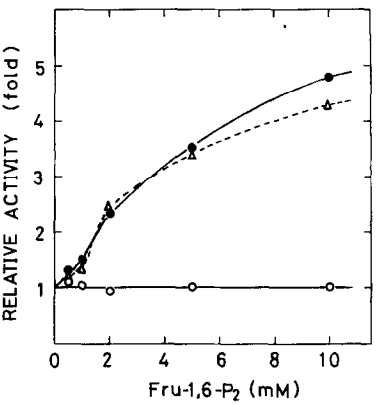


Fig. 2. Reversibility of desensitization to Fru-1,6-$P_2$. Preparation of the modified and reactivated enzymes was described in the text. The assay mixture contained indicated concentrations of Fru-1,6-$P_2$ and the following enzymes: ●, native enzyme (4 μg protein); O, butanedione modified enzyme (8 μg protein) with residual activity of 5%; Δ, reactivated enzyme (8 μg protein) with 80% activity of the native enzyme.

GTP. The modification also caused a partial desensitization to CoASAc, laurate and L-aspartate. In consideration of the fact that the *Km* value of this modified enzyme for PEP was almost the same as that of the native enzyme, the desensitization to Fru-1,6-$P_2$ and GTP was not due to the alteration of the *Km* value for PEP. These results indicate that the amino acid residue(s) responsible for the regulatory interaction with Fru-1,6-$P_2$ or GTP is distinct from that involved in the catalytic interaction with the substrate.

Reversibility of the butanedione-inactivation and desensitization was examined according to the method established by Riordan (8). The enzyme was inactivated with 5 mM butanedione to 5% of the initial enzymatic activity and then the mixture was passed through a small column of Sephadex G-50 (0.6 x 12.0 cm) equilibrated with 100 mM Tris-$H_2SO_4$ buffer (pH 8.5). When the eluted protein was incubated at 30°C, 80% of the initial enzymatic activity was recovered in 120 min. The effector sensitivities of this reactivated enzyme were examined. Although the modified enzyme was not activated at all by increasing the concentration of Fru-1,6-$P_2$ up to 10 mM, the reactivated enzyme with 80% activity showed almost the same degree of activation as the native enzyme did (Fig. 2). A similar desensitization-resensitization phenomenon was observed with GTP (data not shown).

Butanedione is known to be a highly selective reagent for modification of the arginyl residue in various proteins (8,9). As previously reported (10), PEP carboxylase of *E. coli* W has 49.4 arginyl residues per subunit. Amino

acid analysis of the 90% inactivated PEP carboxylase by butanedione showed that the inactivation was accompanied with modification of 9.0 arginyl residues per subunit without any modification of other amino acids.

It has been established that arginyl residue(s) plays an essential role in a number of enzymes, providing the positive charge so as to recognize and to interact with negatively charged substrates or effectors such as AMP (11), NADH (12) and phosphate esters (13). The present work strongly suggests that the arginyl residues play important roles in the catalytic and regulatory functions of PEP carboxylase.

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