Phosphoenolpyruvate Carboxylase of *Escherichia coli*. Multiple Conformational States Elicited by Allosteric Effectors†

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ABSTRACT: An analysis of the conformational state of phosphoenolpyruvate carboxylase [EC 4.1.1.31] from *Escherichia coli* was performed by examination of influence of allosteric effectors on the rate of the enzyme inactivation by *N*-ethylmaleimide (NEM). The SH group(s) susceptible to NEM modification was essential for catalytic function but not for regulatory ones. The presence of a potent competitive inhibitor, DL-2-phospholactate, protected the enzyme from NEM inactivation, suggesting that the essential SH group(s) is located at or near the active site. L-Aspartate, the allosteric inhibitor, protected the enzyme against NEM inactivation. The extent of the protection was saturable with increasing concentrations of L-aspartate and the concentration required for a half-maximal protection (0.4 mM) was nearly equal to that required for 50% inhibition (0.2–0.4 mM). In contrast, the effects of acetyl-CoA (CoASAc), fructose 1,6-diphosphate, and laurate—the allosteric activators—on the inactivation were weakly protective, ineffective, and accelerative, respectively. The results indicate that the conformational states elicited by these allosteric effectors are different from one another in the reactivity of essential SH group(s) toward NEM. Combined additions of these activators, except for a combination of CoASAc and laurate, strongly protected the enzyme against NEM inactivation, indicating that additional new conformational states were brought about by the binding of different kinds of activators to the enzyme at the same time. The multiplicity of the conformational state was further supported by the fact that the effectors showed protections of varying degrees of extent against heat inactivation of the enzyme. These results indicate that the enzyme can take at least seven conformational states by binding with the allosteric effectors singly and in combinations.

Phosphoenolpyruvate carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] of Enterobacteriaceae is known to play an anaplerotic role by replenishing oxaloacetate to the tricarboxylic acid cycle (Theodore and Englesberg, 1964; Ashworth and Kornberg, 1966) and is known to be one of the characteristic allosteric enzymes whose activities are controlled by a multiplicity of effectors. The enzyme from *Escherichia coli* W is inhibited by L-aspartate (Nishikido *et al.*, 1965, 1968; Izui *et al.*, 1967), and activated by CoASAc[1] (Cánovas and Kornberg, 1965), FDP (Izui *et al.*, 1970a), and by long-chain fatty acids or their CoA derivatives (Izui *et al.*, 1970b). Moreover, the *E. coli* enzyme as well as the corresponding enzyme of *Salmonella typhimurium* (Sanwal *et al.*, 1966), is activated by various organic solvents such as dioxane and alcohols (Katsuki *et al.*, 1967) which are presumed to bind with the enzyme at the site for long-chain fatty acid (Izui *et al.*, 1970b). The existence of four distinct regulatory sites on the enzyme for binding with the above mentioned effectors was shown by the method of genetic desensitization (Morikawa *et al.*, 1971). Since the enzyme is composed of four identical subunits with a molecular weight of 88,200 (Yoshinaga *et al.*, 1970, 1974), each subunit is possibly equipped with a set of four regulatory sites.

In general, the control of catalytic activity by allosteric effectors is considered to be mediated by enzyme protein through changes in its conformation. Therefore, it seemed of particular interest to inquire how many conformational states are accessible to such an enzyme having many allosteric effectors. Several methods have been employed to detect the conformational changes of allosteric enzymes. One of them is to examine the changes in reactivity of particular amino acid residue of the enzyme toward an appropriate chemical reagent, which is caused by binding with allosteric effector. As a typical example, this method was used with success by Gerhart and Schachman (1968) to detect two different conformational states of aspartate transcarbamylase.

As will be described in this communication, SH group(s) of the enzyme susceptible to the NEM modification was found to be essential for catalytic function but not for regulatory one. Accordingly, the change in chemical reactivity of the SH group(s) was expected to be available as a probe for the conformational changes associated with allosteric interactions. Study was conducted to examine the effect of various effectors which were added individually and in combination on the rate of the enzyme inactivation. In addition, the influence of allosteric effectors on the rate of heat inactivation of the enzyme was investigated to obtain further information on the nature of each conformational state. As a result of the experiments, the changes in the chemical reactivity and heat stability of the enzyme were found to occur only under the conditions similar to those in which allosteric effects were detected by kinetic measurements (Izui, 1970).

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[1] Abbreviations used are: CoASAc, acetyl-CoA; FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; NEM, *N*-ethylmaleimide; P-lactate, DL-2-phospholactate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

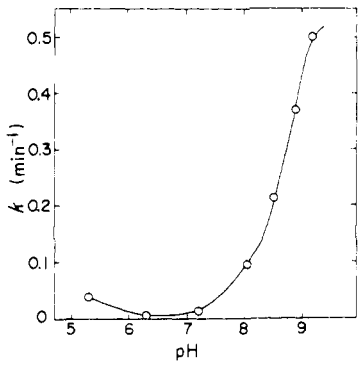

FIGURE 1: pH dependence of the rate constant (k) of NEM inactivation of PEP carboxylase. The enzyme (185 μg of protein) was incubated at 30° with 1 μmol of NEM in 1 ml of 0.1 M Tris-acetate buffer of indicated pH. At proper intervals, 10-μl aliquots were withdrawn and assayed for the residual enzyme activity in the standard assay mixture.

It seems therefore that the observed conformational changes are equivalent or closely linked to those mediating allosteric effects. This communication reports that PEP carboxylase of *E. coli* can take multiple conformational states (at least seven) by binding with the allosteric effectors.

Materials and Methods

Reagents. PEP and P-lactate were synthesized by the methods of Hashimoto and Yoshikawa (1962) with some modification, and of Wagner-Jauregg (1935), respectively. CoASAc was prepared by the method of Simon and Shemin (1953) and was determined according to Tubbs and Garland (1969). Bovine serum albumin, dithiothreitol, FDP, glucose 6-phosphate, NADH, NEM, and Tris were purchased from Sigma, Sephadex G-50 from Pharmacia, malate dehydrogenase [EC 1.1.1.37] from Boehringer-Mannheim, and poly(ethylene glycol) from Daiichi Pharmaceutical Co., Tokyo. All other chemicals were from Nakarai Chemicals Co., Kyoto.

Preparation of Enzyme. PEP carboxylase was prepared from *E. coli* W as described previously (Yoshinaga *et al.*, 1970, 1974), and was stored at 0° in suspension in 60% saturated ammonium sulfate solution. Before use, the enzyme was reactivated by the addition of dithiothreitol (1–2 mM), and the solution was passed through a Sephadex G-50 column equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) to remove ammonium sulfate and remaining dithiothreitol. The enzyme preparation with a specific activity of 35 units/mg of protein (53% pure) was used throughout this study unless otherwise noted.

Assay of Enzyme Activity. The enzyme activity was determined at 30° by following the rate of NADH oxidation at 340 nm in a Hitachi 124 recording spectrophotometer (Yoshinaga *et al.*, 1970). The standard assay mixture contained, in 1 ml, 2 μmol of potassium PEP, 10 μmol of $MgSO_4$, 10 μmol of $KHCO_3$, 10% (v/v) of dioxane, 0.1 μmol of dithiothreitol, 0.15 μmol of NADH, 100 μmol of Tris-H_2SO_4 buffer (pH 8.5), 10 IU of malate dehydrogenase, and the enzyme as indicated. One unit of the enzyme was defined as the amount of oxidizing 1 μmol of NADH/min under the assay conditions. Specific activity was expressed as units/mg of protein. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Measurement of Rates of Enzyme Inactivation by NEM. The enzyme was incubated at 30° with 1 μmol of NEM in 1 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.3) containing effector(s) or other additions when indicated. The buffer used was freed from O_2 by bubbling N_2 gas. At indicated time intervals, 10-μl aliquots of the incubated mixture were withdrawn and immediately added to the standard assay mixture to determine the residual enzyme activity. The inactivation reaction proceeded following pseudo-first-order kinetics and the rate constant (k) was obtained from the slope of semilogarithmic plots on the basis of the well-known relation ($\ln (v/v_0) = -kt$, where v is the residual activity at time t and v_0 is the original activity).

TABLE 1: Protection of PEP Carboxylase against NEM Inactivation by P-lactate[a]

Expt	Addition (mM)			Inactivation Rate Constant k (min^{-1})
	CoASAc	$MgSO_4$	P-lactate	
1	0.5	10	0	0.238
	0.5	10	1.25	0.165
	0.5	10	2.5	0.132
	0.5	10	5.0	0.103
	0.5	10	10.0	0.069
2	0	0	0	0.170
	0	0	2.0	0.189
3	0.5	0	0	0.124
	0.5	0	1.0	0.111
4	0	10	0	0.322
	0	10	1.25	0.357

[a] Experimental conditions were the same as described in Materials and Methods except for the addition of CoASAc, $MgSO_4$, and P-lactate when indicated.

Results

Inactivation of PEP Carboxylase by NEM. The enzyme was inactivated by incubation with NEM in Tris-H_2SO_4 buffer (pH 8.3) at 30°. The inactivation proceeded following pseudo-first-order kinetics since the amount of NEM was much larger than that of the SH group of the enzyme. The almost complete inactivation was provided after 40-min incubation. The inactivation rate was augmented 1.85-fold by the addition of 10 mM Mg^{2+}, one of the necessary components for the catalytic action. Under these conditions, a spontaneous inactivation of the enzyme in the absence of NEM was not seen for 30-min incubation. In addition, essentially no alteration of allosteric properties occurred during the course of the modification. These results indicate that some SH groups susceptible to the NEM modification are essential for the catalytic activity but not for the regulatory activity as reported previously (Teraoka *et al.*, 1972b). The inactivation rate constant was proportional to NEM concentration over a range from 0.2 to 2.0 mM. The rate constant was also markedly dependent on pH (Figure 1) as was often observed in the case of several other enzymes susceptible to NEM inactivation. It was low below pH 7.5 and increased steeply as pH value increased, indicating that an ionized form of the SH group(s) is a reactive species with NEM (Lynen, 1970). Since pK for the inactivation was in a normal range (8.5–9.0), the SH group(s) involved in the NEM inactivation seems to be located in an electrostatically neutral environment of the enzyme protein (Webb, 1963).

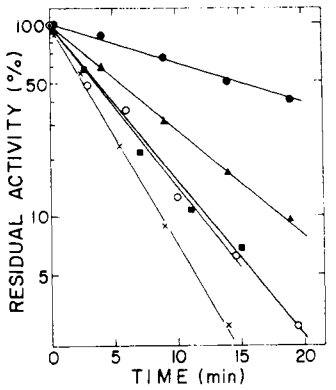

FIGURE 2: Influence of allosteric effectors on NEM inactivation of PEP carboxylase. Experimental conditions were the same as described in Materials and Methods. The enzyme amount used was 75 μg of protein. The inactivation rate constants in the presence of 10 mM L-aspartate (●), 0.88 mM CoASAc (▲), 10 mM FDP (■), 0.5 mM laurate (x), and in the absence of the effector (○) were 0.048, 0.123, 0.189, 0.263, and 0.185 min^{-1}, respectively.

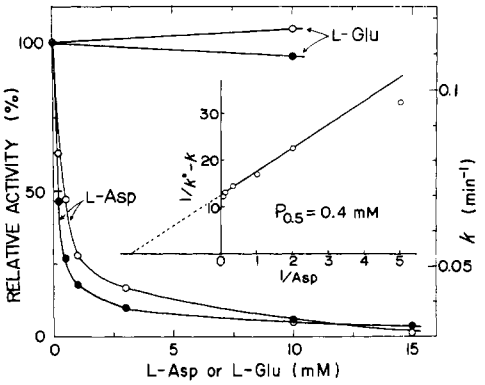

FIGURE 3: Effects of L-aspartate and L-glutamate on the rate constant (k) of NEM inactivation and on the activity of PEP carboxylase: (○) rate constant; (●) enzyme activity. The reaction conditions for the NEM inactivation experiment were the same as described in Materials and Methods except for the presence of L-aspartate or L-glutamate at indicated concentrations. The reaction mixture for the measurement of inhibition by L-aspartate was also the same as described in Materials and Methods except for the concentration of PEP (1 mM), the omission of dioxane, and for the addition of L-aspartate or L-glutamate as indicated. The enzyme amount used was 39 μg of protein in the two experiments. The inset shows double reciprocal plots of ($k^0 - k$) and L-aspartate concentration, where k stands for the rate constant in the presence of varying concentrations of L-aspartate and k^0 for the rate constant in its absence.

Protection against NEM Inactivation by P-lactate. When kinetic measurements were carried out in the modified standard assay mixture which contained 0.44 mM CoASAc instead of dioxane as an activator, P-lactate, an analog of PEP, acted as a potent competitive inhibitor with PEP as in the case of PEP carboxykinase [EC 4.1.1.32] (Miller and Lane, 1968). PEP saturation curves in the presence of various concentrations of P-lactate were always hyperbolic under these conditions. The inhibition constant (K_i) of P-lactate was markedly dependent on the concentration of the allosteric activator owing to the heterotropic interaction between catalytic and regulatory sites (*cf.* Izui, 1970). For example, 2 mM P-lactate gave no significant inhibition in the absence of the activator, though it gave a 50% inhibition in the presence of 0.44 mM CoASAc at PEP concentration of 1 mM. K_i and K_m values in the presence of 0.44 mM CoASAc were 0.90 and 0.69 mM, respectively. In the presence of 10% dioxane instead of CoASAc, K_i and K_m values were 0.46 and 0.54 mM, respectively.

Table I shows the effect of P-lactate on the rate of the NEM inactivation. P-lactate was protective against the inactivation only in the presence of both $MgSO_4$ and CoASAc, and the protecting effect increased with increasing concentrations of P-lactate, exhibiting a tendency to afford a complete protection at infinite concentrations of it (experiment 1). An $F_{0.5}$[2] obtained from the double reciprocal plots of these data was 2.0 mM. Mg^{2+} requirement for the manifestation of protecting effect of P-lactate seems to be due to its role as a bridge between P-lactate and the enzyme as in the case of peanut PEP carboxylase (Miller *et al.*, 1968). These results strongly suggest that the essential SH group(s) is located at or near the active site, or at some other site where the reaction with NEM is hindered by the binding of the enzyme with P-lactate through the conformational change of the enzyme.

Influence of Allosteric Effectors on NEM Inactivation. Effects of the four kinds of the allosteric effectors—L-aspartate, CoASAc, FDP, and laurate—on the rate of the NEM inactivation of the enzyme were investigated (Figure 2). The concentration of each effector added was enough for the manifestation of its maximal effect on the enzyme activity. L-Aspartate, the allosteric inhibitor, strongly protected the enzyme against the inactivation, namely, it decreased the rate constant to about one-fourth that of the control. As shown in Figure 3, the inactivation rate constant decreased in a hyperbolic manner with increasing concentrations of L-aspartate and showed a tendency to reach a lower limited value at its infinite concentrations. A $F_{0.5}$ value of 0.4 mM obtained from the figure was in fairly good agreement with the inhibition constant of L-aspartate ($I_{0.5}$ = 0.2 mM). Since the $I_{0.5}$ value was substantially independent of the concentration of added PEP so far as it was lower (<2 mM) than the half-saturation concentration (25 mM), the $F_{0.5}$ value obtained in the absence of PEP could be compared directly with the $I_{0.5}$ value obtained in the presence of 1 mM PEP. The rationale of this comparison will be given later. The protecting effect was specific for L-aspartate, since L-glutamate, an analog of L-aspartate, scarcely affected the inactivation rate constant as well as the catalytic activity. These results imply that the observed change in the reactivity of essential SH group(s) of the enzyme reflects its conformational change induced by the interaction between L-aspartate and the enzyme at the allosteric site.

Figure 2 further shows effects of the activators on the NEM inactivation. CoASAc weakly protected the enzyme against the inactivation. A $F_{0.5}$ value was found to be 0.65 mM from the curve showing the relationship between the rate constant and CoASAc concentration (not shown). FDP had no effect on the inactivation. On the other hand, laurate accelerated the inactivation. Since PEP carboxylase was not inactivated by laurate in the absence of NEM within 30 min, the accelerating effect of laurate on the NEM inactivation was not due to the labilization of the enzyme through its detergent action. However, it was unable to obtain a $F_{0.5}$ value for laurate because the extent of increase in the rate constant of the NEM inactivation was too small for quantitative analysis.

Previously, kinetic studies showed that L-aspartate strengthened the sigmoidal nature of the FDP-activation

[2] The notation was defined as a ligand concentration required for a half-maximal effect on NEM inactivation, or on heat inactivation of the enzyme.

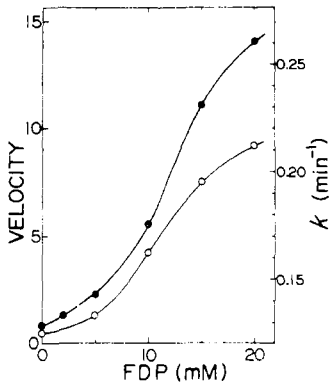

FIGURE 4: Effect of increasing concentrations of FDP on the rate constant (k) of NEM inactivation and on the activity of PEP carboxylase in the presence of L-aspartate: (○) rate constant; (●) enzyme activity. Experimental conditions in the NEM inactivation were the same as described in Materials and Methods except for the additions of 0.5 mM L-aspartate and indicated concentrations of FDP. The enzyme amount used was 107 μg of protein. The reaction mixture for the assay of activation by FDP was the same as described in Materials and Methods except for the concentration of PEP (1 mM), the omission of dioxane, and for the additions of L-aspartate (0.5 mM) and FDP at indicated concentrations. The enzyme amount used was 1.1 μg of protein. The reaction velocity was represented as nanomoles of NADH oxidized per minute.

curve (Izui *et al.*, 1972, unpublished data) and of the CoASAc-activation curve (Izui *et al.*, 1967). As obviously seen in Figure 4, the increasing concentrations of FDP showed a tendency to release the protecting effect caused by 0.5 mM L-aspartate, and the FDP-k curve as well as activation curve by FDP was markedly sigmoidal. The fact that the heterotropic interaction between FDP and L-aspartate was observed on their effect on the rate constant again indicates a close correlation between changes in k and in conformation arising from allosteric interaction.

Effect of Combined Addition of Activators on NEM Inactivation. Since L-aspartate decreased the enzyme affinity for CoASAc, FDP, and laurate (Izui *et al.*, 1972, unpublished data), it seemed improbable that the inhibitor and one of the activators bind with the enzyme at the same time. In contrast, one of the three activators increased the affinity of the other activators so remarkably that all the activators were expected to be able to bind with the enzyme at the same time. From this point of view, studies were performed

TABLE II: Effect of Combined Addition of Activators on the Rate Constant (k) of NEM Inactivation of PEP Carboxylase.[a]

Expt	Effector Added	k (min^{-1})
1	None	0.159
2	FDP (10 mM)	0.170
3	CoASAc (0.41 mM)	0.136
4	Laurate (0.3 mM)	0.184
5	FDP + CoASAc	0.017
6	FDP + laurate	0.056
7	CoASAc + laurate	0.147
8	FDP + CoASAc + laurate	0.017
9	G6P (10 mM) + CoASAc	0.149

[a] Experimental conditions were the same as described in Materials and Methods except for the amount of the enzyme used (85 μg of protein). The effectors were added in final concentrations as indicated. G6P = glucose 6-phosphate.

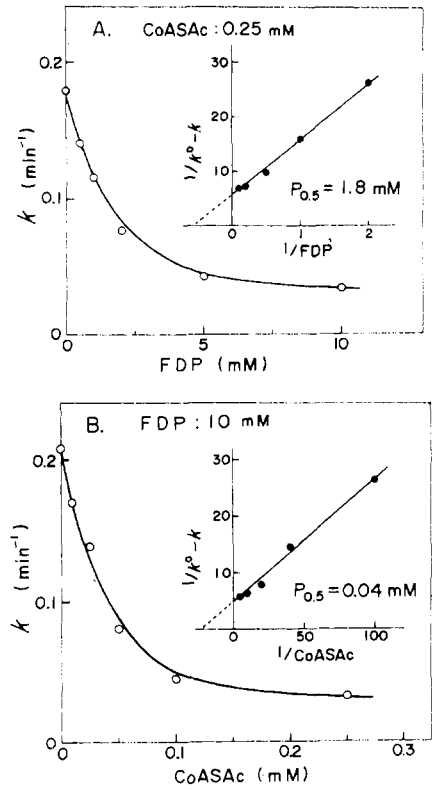

FIGURE 5: Dependence of the rate constant (k) of NEM inactivation of PEP carboxylase on FDP and CoASAc concentration in their combined additions. Experimental conditions were the same as described in Materials and Methods except for the additions of indicated concentrations of FDP and 0.25 mM CoASAc (A) or for the additions of indicated concentrations of CoASAc and 10 mM FDP (B). The enzyme amount used was 190 μg of protein. The insets represent double reciprocal plots of ($k^0 - k$) and activator concentration, where k stands for the rate constant in the presence of varying concentrations of activator and k^0 for the rate constant in its absence.

on the conformational state of the enzyme in the presence of two or three activators in combination, and the results are summarized in Table II. Three combinations of the activators, except a combination of CoASAc and laurate which had no effect on the inactivation (experiment 7), strongly protected the enzyme against the NEM inactivation (experiments 5, 6, and 8). Replacement of FDP by glucose 6-phosphate in the combination of FDP and CoASAc resulted in a much weaker protection than the original combination (experiment 9). This indicated a high structural specificity of FDP in the additional protecting effect to the sole effect of CoASAc. In order to verify that such a protecting effect of FDP in combination with other activator(s) against the NEM inactivation was due to the conformational change of the enzyme associated with allosteric interaction, the inactivation rate constant was measured at varying concentrations of FDP and CoASAc. In the presence of 0.25 mM CoASAc, the effect of FDP on the NEM inactivation rate depended on its concentration, showing a $F_{0.5}$ of 1.8 mM (Figure 5A). In the presence of 10 mM FDP, likewise, the inactivation rate constant decreased with increasing concentrations of CoASAc, showing a $F_{0.5}$ of 0.04 mM (Figure 5B). Since a marked change in the $F_{0.5}$ value for CoASAc (from 0.65 to 0.04 mM) caused by the addition of FDP seems due to the heterotropic interaction between CoASAc and FDP, it is presumed that a new conformational state was induced in the enzyme protein upon binding with both CoASAc and FDP. As in the case of FDP *plus*

TABLE III: Influence of Effectors on the Rate Constant (k) of Heat Inactivation of PEP Carboxylase.[a]

Effector Added	k (min^{-1})
None	0.323
L-Aspartate (10 mM)	0.057
FDP (10 mM)	0.119
CoASAc (0.41 mM)	0.320
FDP + CoASAc	0.109

[a] The solutions containing the enzyme (122 μg of protein) and various effectors in 1 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.3) were heated in a water bath of 51° after 1-min preincubation at 35°. At proper intervals, 50-μl aliquots were withdrawn and added to 0.1 ml of the ice-cold buffer containing 2 mM dithiothreitol, and then the enzyme activity was determined.

CoASAc, protecting effects of FDP *plus* laurate, and CoASAc *plus* FDP *plus* laurate against the NEM inactivation are presumed to reflect conformational changes of the enzyme.

Influence of Allosteric Effectors on Heat Inactivation of PEP Carboxylase. In order to detect conformational change of the enzyme by another method, influences of the effectors on heat inactivation of the enzyme were examined (Table III). The inactivation proceeded following first-order kinetics. L-Aspartate, FDP, and CoASAc were markedly protective, moderately protective, and ineffective against the inactivation, respectively. The results were compatible with our preliminary observation (Izui *et al.*, 1970a). Addition of CoASAc to the incubation mixture containing FDP caused no further significant change in the protection. Although laurate enhanced the inactivation rate to sixfold (not shown), the effect might have been due to its detergent action which predominates at so high temperature employed. The rate constant of heat inactivation was decreased to one-sixth by the addition of 10 mM L-aspartate and to one-third by the addition of 10 mM FDP. Furthermore, the changes in the rate constant were saturable with increasing concentrations of L-aspartate and of FDP, showing $F_{0.5}$ values of 3.4 and 2.0 mM, respectively. In contrast, L-glutamate, an analog of L-aspartate, and glucose 6-phosphate, an analog of FDP, were scarcely effective for the protection. These results indicate that the protecting effects of L-aspartate and FDP on the heat inactivation were caused through conformational change associated with allosteric interaction. Therefore, conformational states elicited by L-aspartate, FDP, and CoASAc seem to be different from one another at 51°.

Sucrose Density Gradient Centrifugation of PEP Carboxylase. The native- and NEM-modified enzyme were analyzed by sucrose density gradient centrifugation (Figure 6) to detect an alteration in the subunit structure associated with the NEM modification, if any. The protein peak of the NEM-modified enzyme with a residual activity of 15% appeared in the same fraction as that of the native enzyme, though it had a trailing shoulder. The result indicates that substantially neither dissociation to subunits nor aggregation of the enzyme occurred by the NEM modification.

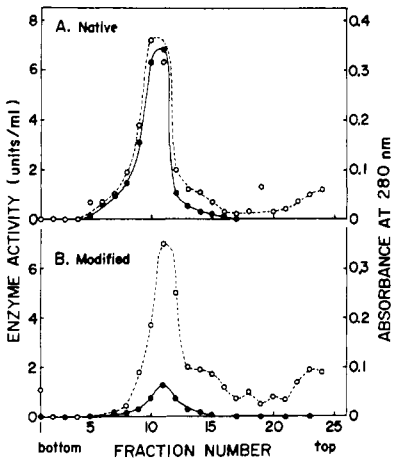

FIGURE 6: Sucrose-density gradient centrifugation of native (A) and NEM-modified PEP carboxylase (B). The pure enzyme (1 mg) with a specific activity of 66 units/mg of protein was incubated with 2 mM NEM in 1 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.3). Dithiothreitol (3 μmol) was added to the mixture after 3 min. The modified-enzyme solution (residual activity of 15%) and the native-enzyme solution (1 mg of protein in 1 ml of the buffer) were put in dialysis bags and concentrated with the aid of poly(ethylene glycol) to a final volume of 0.2 ml. Each solution (0.1 ml) was layered on a 4.8-ml gradient of 5–20% (w/v) sucrose solution containing 0.1 M Tris-H_2SO_4 buffer (pH 8.0). Centrifugation was performed for 8 hr at 40,000 rpm and 15° in a RPS-50 rotor in a Hitachi Model 65P centrifuge. Three drops per fraction were collected from the bottom of the tube. Fractions diluted fivefold were used for the measurement of absorbance at 280 nm (○), and for the assay of the enzyme activity (●). The assay mixture was the same as described in Materials and Methods except for the omission of dioxane. The scale of the ordinate is corrected for the dilution of the sample.

Discussion

SH Groups of PEP Carboxylase. The results presented in this paper indicate that PEP carboxylase was completely inactivated by NEM and the rate constant of the inactivation was significantly affected to various extents by the allosteric effectors. Our recent studies on SH groups of the enzyme have revealed the following facts (Yoshinaga *et al.*, 1974): (a) DTNB which reacts with two of eight SH groups per subunit of the enzyme affects neither catalytic activity nor allosteric property of the enzyme; (b) NEM reacts with four SH groups. Two of them are susceptible to the DTNB modification, and either or both of the other two SH groups are essential to the enzyme activity; (c) the remaining four SH groups are not reactive with DTNB or NEM, unless the enzyme is unfolded with sodium dodecyl sulfate. As observed by Cánovas and Kornberg (1966), and Smith (1968), *p*-mercuribenzoate partially and reversibly inactivated the enzyme (not shown). These observations indicate that NEM, DTNB, and *p*-mercuribenzoate react with SH groups in different manners from one another and give different effects on the enzyme properties as, for example, observed on carbamoyl phosphate synthetase (Novoa *et al.*, 1966). Although conclusive observation is still lacking to decide whether the essential SH group(s) was located at or near the active site, or at some other site, the former possibility seems more likely in view of the following observations: (a) the almost complete protection was afforded by a substrate analog, P-lactate; (b) effect of the NEM modification on the enzyme structure seemed to be localized because neither subunit structure (Figure 6) nor allosteric properties (Teraoka *et al.*, 1972b) were altered significantly upon the modification; (c) acceleration of the NEM inactivation by Mg^{2+} was attributable to a decrease in pK value of the essential SH group(s), which was brought about by the proximity effect (Webb, 1963) of positive charge of Mg^{2+} bound to the active site (Miller *et al.*, 1968).

TABLE IV: Correlation between $F_{0.5}$ and Inhibition Constant ($I_{0.5}$) or Activation Constant ($A_{0.5}$).[a]

Effector	$I_{0.5}$ or $A_{0.5}$ (mM)	$F_{0.5}$ (mM) Obtained by NEM Inactivation	$F_{0.5}$ (mM) Obtained by Heat Inactivation
L-Aspartate	0.2 0.4[b]	0.40	3.4
CoASAc	0.2–0.4	0.65	[c]
FDP	4 6	[c]	2.0
Laurate	0.05	[d]	[d]
CoASAc (+ 10 mM FDP)	0.05	0.04	[c]
FDP (+0.25 mM CoASAc)	1.0	1.80	2.0

[a] $I_{0.5}$ and $A_{0.5}$ were estimated from double reciprocal plots of effector concentration and initial velocity. The initial velocity was measured under the standard assay conditions except for the concentration of PEP (1 mM) and for the omission of dioxane. These values were not significantly affected even when PEP concentration was reduced to 0.5 mM. [b] Indicates $I_{0.5}$ value. [c] No effect. [d] Not determined.

Conformational Changes of PEP Carboxylase. In this study the reactivity of essential SH group(s) toward NEM was used as a probe for the detection of conformational change of the enzyme. The effects of the allosteric inhibitor and three kinds of activators which were added separately and in combination on the reactivity were quantitatively analyzed. In Table IV are summarized $F_{0.5}$ values in comparison with inhibition constants ($I_{0.5}$) and activation constants ($A_{0.5}$). There is considerably close correlation between them, indicating that the conformational changes associated with allosteric interactions were detected by the methods employed here.

L-Aspartate, the allosteric inhibitor, strongly protected the enzyme against the NEM inactivation, showing an $F_{0.5}$ of 0.4 mM. The protective effect was specific for L-aspartate and the shape of the curve representing the dependence of the inactivation rate constant on L-aspartate concentration mimicked the L-aspartate-inhibition curve. Since no cooperativity of L-aspartate is seen in both curves, the fairly good agreement of $F_{0.5}$ value with $I_{0.5}$ value for L-aspartate is interpreted in a classical manner as follows. When NEM concentration is much more excess than that of the enzyme, the observed inactivation rate constant (k) of pseudo first order is given by

$$k = (k^0[E] + k_I^0[EI])/([E] + [EI]) \quad (1)$$

where E, I, and EI, stand for the free enzyme subunit, L-aspartate, and the enzyme–L-aspartate complex, respectively, and k^0 and k_I^0, for the rate constants of the reactions with NEM of E and EI, respectively. The dissociation constant of the enzyme–L-aspartate complex (K_d) which is considered to be nearly equal to $I_{0.5}$ is given by

$$I_{0.5} \approx K_d = [E][I]/[EI] \quad (2)$$

From eq 1 and 2, eq 3 can be derived. Accordingly, if $F_{0.5}$

$$k - k^0 = (k_I - k^0)[I]/(K_d + [I]) \quad (3)$$

for L-aspartate obtained from double reciprocal plots of ($k - k^0$) *vs.* [I] (see Figure 3) was close to $I_{0.5}$, the protection caused by L-aspartate could be attributed to the conformational change of the enzyme associated with the allosteric interaction. Although the same relation was expected to hold in the analysis of heat-inactivation experiments, the obtained $F_{0.5}$ value (3.4 mM) was about tenfold higher than the $I_{0.5}$ value (Table IV). This apparent disagreement may be reconciled when the temperature dependence of the $I_{0.5}$ value is taken into consideration, namely, an imaginary $I_{0.5}$ value at 51° estimated by linear extrapolation of the plots of $I_{0.5}$ *vs.* T^{-1} (Yoshinaga *et al.*, 1972, unpublished data) is about 3.3 mM which is very close to $F_{0.5}$ value obtained from the inactivation kinetics at 51°.

When the ligand exhibits a cooperativity, on the other hand, the above described simple equation does not hold. In such a case, a more complicated relation would be necessary. Thus, even if the $F_{0.5}$ value does not accord with the $A_{0.5}$ (or $I_{0.5}$) value, it could not necessarily be concluded that change in rate constant does not reflect conformational change of the enzyme linked with allosteric interaction. $F_{0.5}$ represents a half-saturation concentration in a state function, while $A_{0.5}$ (or $I_{0.5}$) represents that in a saturation function. It follows that these two functions accord or do not accord with each other, depending on the molecular mechanism of the allosteric transition (*cf.* Gerhart and Schachman, 1968). In this study, the above described rationale seems to hold for most cases in which a hyperbolic relationship exists except for the case in which a sigmoidal relationship exists as observed in Figure 4.

As for the effects of the activators on the rate of enzyme inactivation by NEM, CoASAc, FDP, and laurate, they were weakly protective, ineffective, and accelerative, respectively (Figure 2). Since the three activators affected differently the reactivity of the essential SH group(s) in spite of their similar stimulating action on the catalytic activity, the conformational states induced by each of the activators were supposed to be different from one another. The $F_{0.5}$ value for CoASAc was estimated to be 0.65 mM which was close to its $A_{0.5}$ value. The effect of laurate was significant but the $F_{0.5}$ value for laurate was unable to be determined owing to its small effect on the inactivation rate constant. Although CoASAc as well as L-aspartate showed a protecting effect on the NEM inactivation, the conformational states induced by them are considered to be different from each other at least in the structure around the essential SH group(s) because of the difference in their maximal extents of the protection, and of their opposite effects on the catalytic activity. It seems, from these observations, that the enzyme is able to take at least five different conformational states by binding with one of these four allosteric effectors in addition to the unbound state.

Addition of the activators in combination with FDP, namely FDP *plus* CoASAc, FDP *plus* laurate, and FDP *plus* CoASAc *plus* laurate, caused a marked protection against the NEM inactivation (Table II), and the protecting effect increased with increasing concentrations of the effectors. In the combination of FDP and CoASAc, the curves representing the relation between the inactivation rate constant and the effector concentration were hyperbolic (Figure 5) and a close correlation was found between $F_{0.5}$ and $A_{0.5}$ values (Table IV). In contrast, a combined addition of CoASAc and laurate caused no effect on the rate of the NEM inactivation, indicating that also the conformational state induced by them is distinct from the states induced by the single addition of these effectors. Therefore, the conformational states elicited by addition of

the activators in combination with FDP seem to be different not only from the state induced by the combination of CoASAc and laurate but also from each state induced by the individual effectors. However, it remains unsolved whether or not the conformational states induced by the three combinations of activators with FDP are different from one another.

Against heat inactivation of the enzyme, L-aspartate, FDP, and CoASAc were strongly protective, moderately protective, and ineffective, respectively (Table III). It is likely that L-aspartate as well as FDP converts the enzyme to a relatively more stable state against the heat inactivation. These results offer additional and complemental evidence for the multiplicity of conformational state.

Finally, preliminary experiments by the use of sucrose density gradient centrifugation showed that the sedimentation coefficient of the native enzyme was unaltered by the single or combined addition of the effectors (Izui, 1973). Therefore, it seems likely that the influence of allosteric effectors on the rate constant of NEM and heat inactivation was not due to a secondary effect associated with a gross alteration in the subunit structure but rather due to a subtle conformational change of the subunit itself.

Multistate Model. Previously the "three-state model" (T'- ⇌ T- ⇌ R-state) was proposed for PEP carboxylase in order to explain the kinetic data (Izui, 1970). In the model, T'-state denoted the conformational state of the enzyme which was induced by L-aspartate, the inhibitor, and had no affinity for PEP, one of the substrates, and T-state denoted the state which was predominant in the absence of the effector and was catalytically active. R-state denoted the state which was induced by the activator(s) and had a much higher affinity for PEP than T-state. The results presented in this paper not only support the validity of this model more directly, but also indicate that R-state can be subdivided into at least five conformational states which are distinguishable from one another. The "multistate model" thus obtained is schematically illustrated in Figure 7 in reference to the "three-state model." It should be emphasized that the activators added singly and in combination do not necessarily induce the enzyme to the same predestinate conformational state but rather to multiple distinct states.

A concept of multiple (more than three) conformational states which are functionally distinct from one another has been proposed on the basis of kinetic studies on several allosteric enzymes, such as ribonucleoside diphosphate reductase (Larsson and Reichard, 1966), deoxycytidylate aminohydrolase (Kirtley and Koshland, 1967), isocitrate dehydrogenase (Kirtley and Koshland, 1967), and PEP carboxylase (Izui, 1970). On the other hand, on glutamine synthetase of *E. coli* which is known to have eight specific effectors (Shapiro and Stadtman, 1967), Rubin and Changeux (1966) suggested that the kinetic data were explainable in terms of the nonexclusive two-state model of Monod *et al.* (1965). In the case of PEP carboxylase, the multiplicity of conformational state observed seems to be more favorably explained in terms of the model of Koshland *et al.* (1966) which was later developed by Kirtley and Koshland (1967), rather than in terms of the model of Monod *et al.* (1965).

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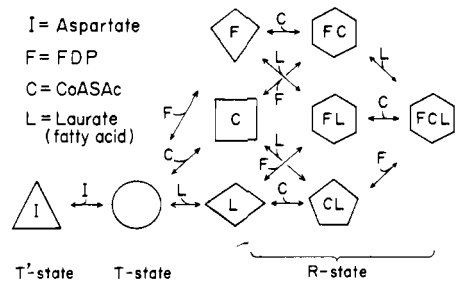

FIGURE 7: Proposed model for the multiple conformational states of PEP carboxylase elicited by allosteric effectors. Geometrical figures—triangle, circle, square, *etc.*—indicate respective conformational states of the enzyme, and for simplicity, indication of subunit structure is deleted. Thus, it follows that the states represented by the same figures are not distinguishable and those represented by the different figures are distinguishable from one another. The capital letter(s) in the figure denotes the effector(s) bound with the enzyme. The denotations of T'-, T-, and R-states are described in the text.

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Purification and Properties of a Neutral Protease from Rat Liver Chromatin†

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ABSTRACT: Rat liver chromatin contains a neutral protease with a marked preference for chromosomal proteins as substrate. The enzyme has been purified about 700-fold. It has a molecular weight of 200,000 with two subunits. It is inhibited by phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate. The enzyme requires divalent ions as activators. The isolated enzyme appears to be similar to that responsible for the endogenous degradation of chromosomal proteins. The susceptibility of the five histone fractions to proteolysis is dependent upon whether the histones are complexed with DNA. In the intact nucleohistone, four major histones are rather resistant to proteolytic attack while histone I is rapidly attacked. If histones are freed from DNA, all the histone molecules are attacked at about the same rate except histone I, which is degraded more slowly than the other histones.

It has been shown previously that isolated chromatin contains protease activity capable of degrading histones in chromatin (Furlan *et al.*, 1968; Panyim *et al.*, 1968; Garrels *et al.*, 1972). Much evidence suggests that association of histones with DNA prevents transcription (Shih and Bonner, 1970; Smart and Bonner, 1971). A possible role for chromatin-bound protease in removing histones from DNA during spermatogenesis has been suggested by Marushige and Dixon (1971). High substrate specificity is implied in this case since the protease must degrade histones without affecting protamines which replace histones in the sperm nucleus. Small heterogeneous acid soluble histone fragments were found in trout testis chromatin late in the transformation from nucleohistone to nucleoprotamine. It is also shown that histones become acetylated and phosphorylated during their replacement (Marushige and Dixon, 1969; Sung and Dixon, 1970; Candido and Dixon, 1972). Thus in spermatogenesis histones may be removed from DNA by proteolytic degradation; minor modifications may render the histones susceptible to such digestion. If the same hypothetical mechanism occurs in removal of histones during gene derepression, then the responsible protease would be an enzyme of biological significance. We have therefore purified this enzyme to homogeneity in an attempt to set the background for future studies on its role in gene regulation.

Materials and Methods

Frozen rat liver was from Pel-Freeze Biologicals, Inc.; poly(L-arginine) (MW 40,000) from Pilot Chemicals, Inc.; poly(L-lysine) (MW 5900) from Miles-Yeda Ltd.; salmon protamine sulfate, egg-white lysozyme, and bovine serum albumin from Sigma Co.; Bio-Rex 70 (200–400 mesh, sodium form), Bio-Gel A-50 (50–100 mesh), calcium phosphate gel, from Bio-Rad Lab; Sepharose 6B, Sephadex G-100, and QAE-Sephadex A-25 from Pharmacia Fine Chemicals; and human γ-globulin (fraction II) and ovalbumin (nonenzymic protein molecular weight markers) from Schwarz/Mann. *Escherichia coli* β-galactosidase was obtained from Worthington Biochemical Corp. Amicon PM-10 ultrafiltration membranes were from Amicon Corp. Dansyl chloride was from Pierce Chem. CO. Polyamide layer sheet was from Gallard-Schlesinger Chem. Corp.

Preparation of Nuclei. Nuclei were prepared by a modification of the method of Blobel and Potter (1966). The yield of nuclei based on the recovery of DNA was 50–70%.

In some instances as indicated in the text, the resulting

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