Biochimica et Biophysica Acta, 452 (1976) 566—579
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BBA 67985

PHOSPHO*ENOL*PYRUVATE CARBOXYLASE OF *ESCHERICHIA COLI*

STUDIES ON MULTIPLE CONFORMATIONAL STATES ELICITED BY ALLOSTERIC EFFECTORS WITH A FLUORESCENT PROBE, 1-ANILINO-NAPHTHALENE-8-SULFONATE

TAKEO YOSHINAGA *

Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606 (Japan)

(Received April 8th, 1976)

Summary

Conformational change of phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) induced by allosteric effectors was investigated using a hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS). Kinetic experiments suggested that ANS binds with the enzyme at the sites which are not involved in the catalytic and regulatory functions, though it partially inhibits the enzyme activity with half-saturation concentration ($S_{0.5}$) of 38.5 μM. Binding experiments showed that a maximum of 2 mol of ANS are able to bind with 1 mol of the enzyme subunit presumably with an equal dissociation constant to each other (34.5 μM). Fluorescence emission of ANS was markedly increased by binding with the enzyme. L-Aspartate, the allosteric inhibitor, and CoASAc and fructose 1,6-bisphosphate (Fru-1,6-P_2), the allosteric activators, produced various degrees of change in fluorescence, when added singly or in combinations. The changes were shown to be attributable to the allosteric interactions between the enzyme and effectors from some criteria such as structural specificity, half-saturation concentrations, and heterotropic-homotropic interactions of the ligands. It was concluded from these analyses that the enzyme can be in at least four conformational states which are distinct from each other. Especially noteworthy is the finding that the enzyme, upon simultaneous binding of CoASAc and Fru-1,6-P_2, takes a new conformation which is entirely different from those induced by sole binding of each effector. In addition, the hetero-

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* Present address: Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606 (Japan).

Abbreviations: CoASAc, acetyl-coenzyme A; Fru-1,6-P_2, fructose 1,6-bisphosphate; ANS, 1-anilinonaphthalene-8-sulfonate.

tropic interaction between the activator and the inhibitor was observed through conformational change by the ANS method, as observed in the kinetic studies.

Introduction

Phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) of the Enterobacteriaceae is known to be one of the characteristic allosteric enzymes which are controlled by multiple effectors. The enzyme from *Escherichia coli* W has a tetrameric structure with identical subunits of molecular weight 88 200 [1,2]. It is inhibited by L-aspartate [3,4] or L-malate [3,4], and is activated by CoASAc [5], Fru-1,6-P_2 [6], and by long-chain fatty acids or their CoA derivatives [7]. Moreover, the *E. coli* enzyme as well as the enzyme of *Salmonella typhimurium* [8] is activated by various organic solvents such as dioxane and alcohols [9] which are inferred to exert their action through binding at the site for fatty acid [7]. The existence of four distinct regulatory sites on each subunit of the enzyme for binding with these effectors was inferred by the method of genetic [10] and chemical [11] desensitization.

It is now widely accepted that allosteric effectors exert their regulatory action on an enzyme by causing conformational change of the enzyme protein, though the precise mechanism of the change is not known. On the allosteric enzyme with multiple effectors such as enteric phospho*enol*pyruvate carboxylase and ribonucleoside diphosphate reductase (EC 1.17.4.1) [12], however, the question has arisen: how many conformational states are accessible to the enzyme upon binding with multiple effectors?

Previous studies from our laboratory presented several lines of evidence for the multiplicity of conformational states of *E. coli* phospho*enol*pyruvate carboxylase. The main method was chemical modification in which the reactivity of essential amino acid residue(s) at the catalytic site towards a chemical reagent was employed as a probe for detection of the conformational changes. As a result of the experiments, it was suggested that the enzyme might be able to be in at least seven different conformational states upon binding with the effectors [13].

In the present study, ANS [14—18] was used as another probe for detection of the conformational changes. Since ANS seemed to bind with the enzyme at sites different from either catalytic or allosteric sites, as will be stated, it was expected to be possible to obtain information about the conformational changes through the interaction of the enzyme with ANS at portions different from either site. The fluorescence of ANS bound with phospho*enol*pyruvate carboxylase was found to change variously when the allosteric effectors (L-aspartate, CoASAc, and Fru-1,6-P_2) were added singly or in combinations of the three and the changes were analyzed quantitatively. In order to ascertain that the observed changes reflect the conformational changes associated with the allosteric interaction, the following three criteria were employed: (1) structural specificity of allosteric effectors required for causing changes in fluorescence should be identical with that required for exerting their effects on the enzyme activity; (2) the degree of the change should be saturable with

increasing concentrations of the effector added and the concentration required for half-maximal change should be identical or close to that required for half-maximal activation or inhibition; (3) heterotropic and homotropic interactions of the effectors should be observable through changes in fluorescence as in the case of kinetic studies. As a result of investigations based on these criteria, the multipicity (at least four) of states of the enzyme could be demonstrated.

Materials and Methods

Reagents

ANS, Fru-1,6-P_2, dithiothreitol and Tris (Sigma 7—9) were obtained from Sigma Chemical Co. Coenzyme A was purchased from Boehringer Mannheim, and L-aspartic acid, L-malic acid, lauric acid, and other chemicals, of analytical grade, were from Nakarai Chemicals Co. Ammonium salt of ANS was treated with charcoal in hot water, filtered through a glass filter, and was recrystallized four times from hot water. The crystals were dried in a vacuum desiccator over phosphorus pentoxide and were stored in the dark. Ammonium salt of ANS in water, pH 6.6, showed an ϵ value of $4.95 \cdot 10^3$ $M^{-1}cm^{-1}$ at 350 nm in accordance with the value reported by Weber and Young [18]. Tris was recrystallized from hot water. Quinine sulfate was recrystallized three times from ethanol. Phospho*enol*pyruvate was synthesized by the method of Clark and Kirby [19] with some modifications. CoASAc was prepared and determined according to the methods of Simon and Shemin [20], and of Tubbs and Garland [21], respectively.

Enzyme

Phospho*enol*pyruvate carboxylase was purified to homogeneity from *E. coli* W as described previously [22]. The enzyme preparation, of more than 95% purity, was used throughout the present study. The enzyme was stored at 0°C in suspension in 60%-saturated ammonium sulfate solution containing 0.1 M Tris · HCl buffer, pH 7.4, 10 mM $MgSO_4$, and 10 mM L-aspartate. Prior to use, the enzyme was treated with 1 mM dithiothreitol at 4°C for 1 h and freed from these additions by passing through a column of Sephadex G-50 equilibrated with 0.1 M Tris · H_2SO_4 buffer, pH 8.0. Enzyme concentration was determined from the absorbance at 280 nm with the use of the extinction coefficient ($E_{1\,cm}^{1\,\%}$) of 11.9 [2]. For calculation of molar concentration of the enzyme, the value of 361 000 was used as molecular weight [2].

Enzyme assay

The enzyme was assayed by a spectrophotometric method with a Hitachi model 124 recording spectrophotometer at 30°C as described previously [22]. The standard assay mixture contained the following consitutents in μmol in 1.0 ml: potassium phospho*enol*pyruvate (2), $MgSO_4$ (10), $KHCO_3$ (10), NADH (0.1), Tris · H_2SO_4 buffer, pH 8.0 (75), 10 I.U. of malate dehydrogenase (EC 1.1.1.37), the enzyme, and additions where indicated.

Binding experiment

Binding experiment of ANS with phospho*enol*puruvate carboxylase was

carried out by the method of Fairclough and Fruton [23]. The binding numbers of ANS at concentrations of 10, 20, and 80 μM were obtained as described in the legend to Fig. 1 except for the change of ANS concentration. The binding experiment in the presence of L-aspartate was also carried out in the same manner as described above except for the addition of 5 mM L-aspartate to all the solutions.

Measurement of fluorescence

All fluorescence emission measurements were carried out at room temperature (24°C) in an Hitachi model 204 Spectrophotofluorimeter equipped with a xenon-arc light source. The wavelengths for the excitation and emission were 380 and 485 nm, respectively, unless otherwise indicated. The fluorimeter was standardized with quinine sulfate. All emission intensities are expressed in arbitrary fluorescence units as indicated.

Procedure for fluorimetric titration

2 ml of 0.1 M Tris · H_2SO_4 buffer, pH 8.0, containing 0.75 nmol of the enzyme was placed in a 1-cm cuvette. 5-μl portions of 4 mM ANS solution were added to the enzyme solution successively. After each addition, the solution was mixed gently with a polyethylene rod and then fluorescence intensity was recorded. Correction due to dilution of the enzyme solution with ANS solution was neglected because the proportion of ANS solution did not exceed 2.5% (v/v). In the standard experiment, the fluorescence emission increased along a hyperbolic curve with increasing concentrations of ANS.

Results

Binding of ANS with the enzyme

Binding experiments of ANS with the enzyme were carried out by a gel-filtration method as described in Materials and Methods. Fig. 1 shows a typical example of the elution pattern which was obtained by measurement of ANS concentration of each eluate fraction. Mole numbers of ANS bound with 1 mol of the enzyme were determined at various concentrations of ANS, and the results are shown in Fig. 2. The double-reciprocal plots of binding number vs. ANS concentration gave an essentially straight line (inset of Fig. 2). The maximum binding amount of ANS was estimated to be 7.4 mol per mol of the enzyme and the dissociation constant (K_d) to be 34.5 μM. Since the enzyme is a tetramer [2], maximum of 2 mol of ANS are presumed to be able to bind with 1 mol of the enzyme subunit.

Effect of ANS on catalytic and regulatory properties of the enzyme

The enzyme activity was inhibited to some degree of extent by ANS. The double reciprocal plots of percent inhibition vs. ANS concentration revealed that the inhibition is a partial inhibition type (the maximum extent of inhibition attainable at infinite concentration of ANS was estimated to be 52.5%) and that the concentration required for half-maximal inhibition was 38.5 μM. The inhibition was not of competitive type with phospho*enol*pyruvate, since the inhibition was not weakened but rather strengthened by increasing the

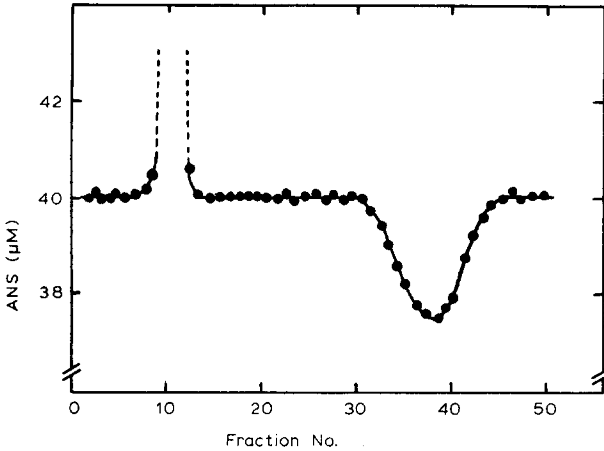

Fig. 1. Gel-filtration pattern of ANS in the binding experiment. The binding number of ANS at a concentration of 40 μM ANS, for example, was obtained by the following procedure. To 0.5 ml of the enzyme (3.3 nmol) insolution in 0.1 M Tris · H_2SO_4 buffer, pH 8.0, was added an equal volume of 80 μM ANS solution in the same buffer, and the mixture was preincubated for 10 min at 28°C. Sephadex G-50 was packed in a column (20 X 1.0 cm) and was equilibrated with 40 μM ANS solution in the buffer. The enzyme ANS mixture was applied to the column and eluted with the same 40 μM ANS solution as that used for the equilibration of the column at a constant flow rate of 1.6 ml per min at 28°C. Fractions of 10 drops (about 0.63 ml) were collected. Each fraction was diluted with 5 ml of ethanol and the fluorescence was measured for determination of ANS concentration. The amount of ANS bound to the enzyme was calculated from the area of the trough of ANS concentration.

concentration of phospho*enol*pyruvate. In addition, the half-saturation concentration of phospho*enol*pyruvate was not increased but was slightly decreased by the presence of 80 μM ANS. These results indicate that the binding sites of ANS are distinct from the catalytic site.

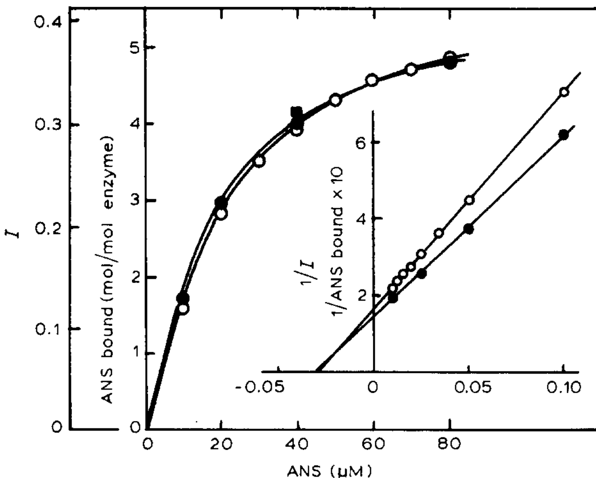

Fig. 2. Relationship between concentration of ANS used for elution and amount of ANS bound with the enzyme, and effects of varying concentrations of ANS on fluorescence emission from the enzyme · ANS complex. The amount of bound ANS was obtained by measurement of the area of the trough in gel filtration. ● and ■ are the amounts of ANS bound with the enzyme in the absence and presence of 5 mM L-aspartate, respectively. ○ is emission intensity (*I*). Inset shows double-reciprocal plots of these data.

Effects of ANS on the allosteric inhibition and activation of the enzyme were investigated. Essentially no effect of 80 μM ANS was observed on the inhibition curves of L-aspartate, and activation curves of CoASAc and Fru-1,6-P_2. Although ANS was supposed to have some affinity to the binding site for long-chain fatty acid owing to its hydrophobic nature, activation by laurate was found to be little affected by the presence of ANS, indicating no competition of ANS with laurate. From these results it could be said that the binding sites for ANS are distinct also from the regulatory sites of the enzyme and that the allosteric properties are essentially unmodified by binding of this probe with the enzyme. Accordingly, it is evident that the present method can detect the conformational change through the interaction of ANS with portions other than the functional sites of the enzyme, differently from the chemical modification method.

Fluorescence of the enzyme · ANS complex

Addition of the enzyme to a dilute solution of ANS (40 μM) in Tris · H_2SO_4 buffer, pH 8.0, resulted in a large increase in fluorescence emission. Fluorescence spectra of the complex without correction are shown in Fig. 3. Excitation maximum was seen at 380 nm (A) and emission peak appeared at 485 nm (B). ANS or the enzyme alone gave a negligibly weak emission under these conditions. Relative magnitude of the fluorescence emission produced from ANS in 0.38 μM phospho*enol*pyruvate carboxylase was 2.9% of that found in ethanol, and was linearly proportional to the enzyme concentration.

Fluorescence of the enzyme · ANS complex was markedly quenched by the addition of L-aspartate in a final concentration of 10 mM without significant

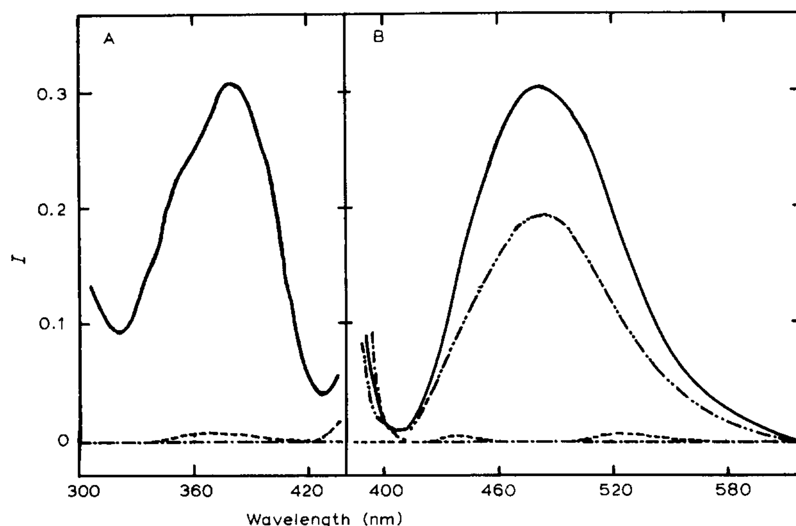

Fig. 3. Fluorescence excitation and emission spectra of ANS bound with the enzyme. Emission was recorded at 485 nm for measurement of the excitation spectrum (A) and excitation was recorded at 380 nm for the emission spectrum (B). The complete mixture contained the enzyme (0.38 μM), 40 μM ANS, and 0.1 M Tris · H_2SO_4 buffer, pH 8.0, in a volume of 2.0 ml. ——, complete mixture; — · —, ANS omitted; - - - - - the enzyme omitted; and — · · —, complete mixture plus 20 μl of 1 M L-aspartate.

change in the position of emission maximum. This suggests that the effector produces a pronounced change in the enzyme conformation.

When the enzyme was titrated with ANS, the fluorescence intensity (I) increased in a hyperbolic manner, showing a tendency to reach a maximum (I_{max}) (Fig. 2). This curve was almost superimposable on the ANS-saturation curve from the binding experiment, and accordingly half-saturation concentration (K_f:36.5 μM) obtained by fluorimetric titration was very close to K_d (34.5 μM) in the binding experiment. Furthermore, K_f was also in close agreement with the concentration required for half-maximal inhibition (38.5 μM). The agreement of the former value with the latter two values indicates that increase in fluorescence emission intensity is due to the binding of ANS with the enzyme itself, and that the two binding sites for ANS on the subunit mentioned in the binding experiment have almost identical affinity for ANS.

Influence of allosteric effectors on fluorescence emission of the enzyme · ANS complex

Fluorimetric titration of the enzyme with ANS was carried out in the presence of various effectors or their analogues which had been known to exert no effect on the activity at the concentrations employed. Concentrations of the effectors were chosen so as to be sufficient for manifestation of their effects on the catalytic activity. The compounds had no absorption at the excitation or the emission region and did not have any effect on fluorescence of ANS in the absence of the enzyme. The double reciprocal plots of I vs. ANS concentration in the presence of various compounds were straight lines. I_{max} and K_f values, and wavelengths for maximum excitations and emissions of the spectra in these experiments are shown in Table I. Addition of L-aspartate or

TABLE I

EFFECTS OF THE ALLOSTERIC EFFECTORS AND THEIR ANALOGUES ON FLUORESCENCE EMISSION FROM THE ENZYME · ANS COMPLEX

The values of Kf and I_{max} in the presence of each ligand were obtained from double-reciprocal plots of I vs. concentration of effector or its analogue. I_{max} denotes the maximum fluorescence intensity of the enzyme · ANS complex attainable at the infinite concentration of ANS, and Kf denotes the concentration of ANS giving $I_{max}/2$.

Effector or its analogue	Concentration (mM)	Spectral peak		K_f (μM)	I_{max} * (%)
		Excitation (nm)	Emission (nm)		
None	—	380	485	36.5	100
L-Aspartate	10	379	487	36.5	62.2
L-Malate	10	380	486	38.5	69.4
L-Glutamate	10	379	485	35.4	100
CoASAc	0.5	379	480	48.1	118
CoASH	0.5	380	483	35.6	97.5
Fru-1,6-P_2	10	380	485	36.5	100
$MgSO_4$	10	381	485	36.5	100
CoASAc + Fru-1,6-P_2	0.5 10	380	484	35.0	69.9

* I_{max} is given as a value relative to I_{max} obtained in the absence of ligand.

L-malate, the allosteric inhibitor, decreased I_{max} by about 40% without any effect on K_f. In contrast, L-glutamate, an analogue of L-aspartate had no effect on either value. CoASAc, one of the activators, increased both I_{max} and K_f by about 20% and 30%, respectively, whereas CoASH had no effect. Fru-1,6-P_2, one of the other activators, did not affect these values. Unexpectedly from the individual effects of CoASAc and Fru-1,6-P_2, combined addition of them resulted in a decrease of I_{max} by about 30% together with a slight decrease in K_f. Mg^{2+}, the essential cofactor, did not affect the fluorescence properties. No variation occurred in fluorescence when the effector was added to ANS or to bovine serum albumin · ANS complex which was used instead of the enzyme · ANS complex as a control. This suggests that the variation of I_{max} and K_f values in these cases reflects specific interaction of the effector with the enzyme · ANS complex. This meets the first of the three criteria for the allosteric transition of the observed variation described in Introduction.

Effects of varying concentrations of L-aspartate on the fluorescence emission of the enzyme · ANS complex

Fluorimetric titration of the enzyme with ANS was carried out in the presence of varying concentrations of L-aspartate. The double reciprocal plots of I vs. ANS concentration revealed that I_{max}, but not K_f, varied with increasing concentrations of L-aspartate. The plots of I_{max} vs. L-aspartate concentration were hyperbolic (Fig. 4) and it was possible to estimate the I_{max} value attainable at infinite concentration of L-aspartate ($I_{max,\infty}$). The concentration required for half-maximal change ($S_{0.5}$) of I_{max} for L-aspartate was 0.55 mM. According to the relation derived by McClure and Edelman [14], this value can be considered to be equal to the dissociation constant of the enzyme · ANS

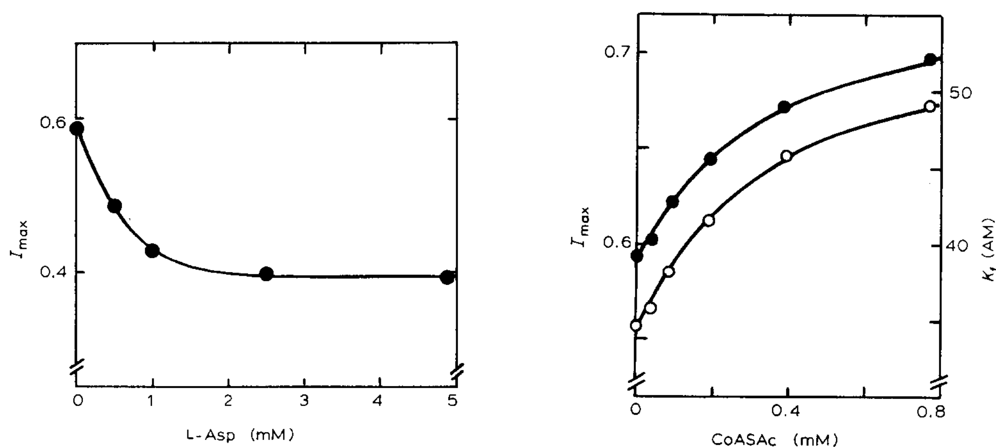

Fig. 4. Effects of increasing concentrations of L-aspartate on maximum emission intensity (I_{max}) of the enzyme · ANS complex. I_{max} was obtained from double-reciprocal plots of I vs. L-aspartate concentration.

Fig. 5. Effects of increasing concentrations of CoASAc on maximum emission intensity (I_{max}) and on half-saturation concentration of ANS obtained by fluorimetric titration (K_f). Experimental procedure was the same as in the experiment using L-aspartate. ●, I_{max}; and ○, K_f.

complex, since the K_f value remained constant at the varying concentrations of L-aspartate employed. (See original paper of these authors for details). The $S_{0.5}$ value of I_{max} was almost equal to the half-inhibition concentration of L-aspartate (Table II), meeting the second criterion for the allosteric transition of the fluorescence change.

The decrease of I_{max} caused by L-aspartate can result either from a decrease in the quantum yield of bound ANS or from a decrease in binding number of ANS with the enzyme. In order to examine which of the two possibilities is valid, measurement of binding number of ANS with the enzyme was carried out by gel-filtration method in the presence and absence of 5 mM L-aspartate. As shown in Fig. 2, the binding number of ANS was not affected by the presence of L-aspartate. This observation strongly suggests that the decrease in I_{max} was due to the decrease in quantum yield of bound ANS.

Effects of varying concentrations of CoASAc on fluorescence emission of the enzyme · ANS complex

The fluorimetric titration of the enzyme with ANS was carried out in the presence of varying concentrations of CoASAc and the data were analysed in the same manner as in the experiment using L-aspartate. As seen from Fig. 5, both K_f and I_{max} increased with increasing concentrations of CoASAc and showed a tendency to reach their maxima at infinite CoASAc concentration. The concentrations required for half-maximal increase in K_f and I_{max} were found from the figure to be 0.38 and 0.39 mM, respectively. The close agreement of these values with the concentration required for half-maximal activation (0.40 mM) meets the second criterion for the allosteric transition for the fluorescence change.

TABLE II

EFFECTS OF ALLOSTERIC EFFECTORS ON THE FLUORESCENCE PROPERTIES OF THE ENZYME · ANS COMPLEX

The concentrations ($S_{0.5}$) of effectors giving half-saturation of I_{max} and K_f values are presented in comparison with those obtained by kinetic studies.

Effector	K_f * (μM)	Relative $I_{max,\infty}$ (%)	$S_{0.5}$ obtained from			Hill coefficient *** (n)
			I_{max} (mM)	K_f (mM)	catalytic activity ** (mM)	
None	36.5	100				
L-Aspartate	36.5	58.8	0.55	— †	0.46	1.0
CoASAc	57.0	127	0.39	0.38	0.4	1.1
CoASAc + 0.2 mM L-Asp	56.7	127	0.59	0.61	MD ††	2.1
CoASAc + 10 mM Fru-1,6-P_2	33.2	67.4	0.04	0.04	0.04	1.0
Fru-1,6-P_2	36.5	100	—	—	3.8	—
Fru-1,6-P_2 + 0.2 mM L-Asp	36.5	100	8.6	—	ND	1.6
Fru-1,6-P_2 + 0.5 mM CoASAc	33.2	67.0	2.4	2.0	2.0	1.0

* The values attainable at infinite concentration of effectors.

** The catalytic activity was measured in the presence of 2 mM phospho*enol*pyruvate.

*** Hill coefficients were obtained from the Hill plots of I_{max} vs. effector concentration.

† Not obtainable because of no change in K_f or I_{max}.

†† Not determined.

Cooperative effect of CoASAc and Fru-1,6-P_2 on fluorescence emission of the enzyme · ANS complex

CoASAc and Fru-1,6-P_2 show a heterotropic cooperativity for *E. coli* phosphoenolpyruvate carboxylase as observed in the kinetic studies, indicating that presence of one activator increases affinity of the other for the enzyme. This means that the two activators are capable of binding with the enzyme simultaneously. As noted in the previous section, combined addition of CoASAc and Fru-1,6-P_2 had an unexpected effect on the fluorescence properties, which was different from the effects of sole addition of these effectors (cf. Table I). The results of titration of the enzyme with ANS in a system containing varying concentrations of one effector in the presence of fixed concentration of the other are given in Fig. 6. In the presence of a fixed concentration of Fru-1,6-P_2 (10 mM), I_{max} was markedly decreased, in a hyperbolic manner, with increasing concentrations of CoASAc and a slight decrease in K_f. The concentration required for half-maximal change ($S_{0.5}$) of I_{max} was 0.04 mM (Fig. 6A) which was one-tenth that obtained in the absence of Fru-1,6-P_2 (Fig. 5). When CoASAc concentration was fixed at 0.5 mM, on the other hand, both I_{max} and K_f decreased with increasing concentrations of Fru-1,6-P_2 (Fig. 6B), showing $S_{0.5}$ values of 2.4 mM and 2.0 mM, respectively. All $S_{0.5}$ values obtained here were in good accordance with those obtained from the kinetic measurements which were carried out under the similar conditions (See Materials and Methods) except for the presence of 2 mM phosphoenolpyruvate (cf. Table II). This meets the third criterion.

Antagonism between L-aspartate and CoASAc or Fru-1,6-P_2 in effect on fluorescence emission of the enzyme · ANS complex

Previous kinetic studies revealed that half-saturation concentration of each

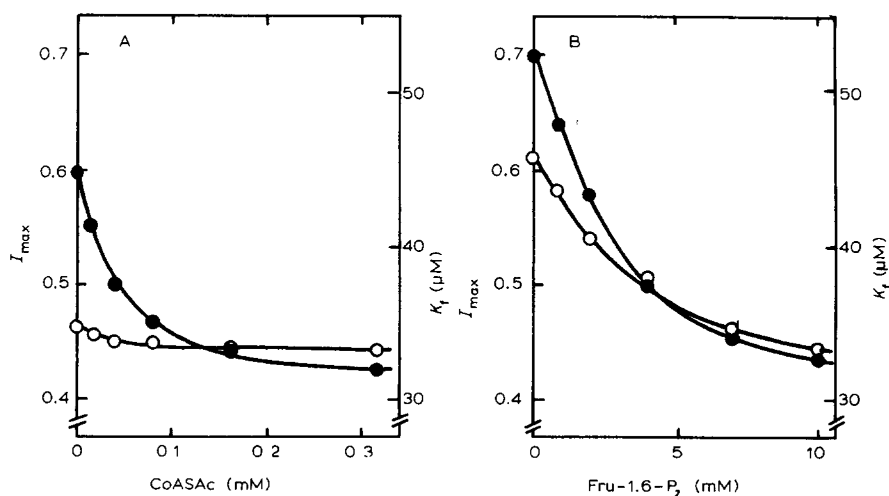

Fig. 6. Cooperative effect of CoASAc and Fru-1,6-P_2 on fluorescence emission from the enzyme · ANS complex. A: Dependence of I_{max} and Kf on the concentration of CoASAc in the presence of 10 mM Fru-1,6-P_2. B: Dependence of I_{max} and Kf on the concentration of Fru-1,6-P_2 in the presence of 0.5 mM CoASAc. ●, I_{max}; and ○, Kf.

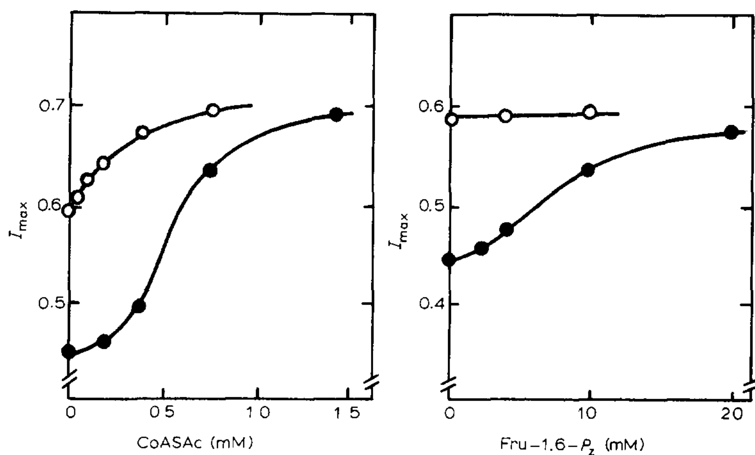

Fig. 7. Effects of increasing concentrations of CoASAc (A) and Fru-1,6-P_2 (B) on maximum emission intensity (I_{mas}) in the presence (●) and absence (○) of L-aspartate 0.2 mM.

activator was increased by the addition of L-aspartate and shape of its saturation curve was changed from hyperbolic to sigmoidal one [24]. Essentially the same response could be observed through the changes of fluorescence properties. As shown in Fig. 7, quenching effects of 0.2 mM L-aspartate were gradually released by increasing the concentrations of CoASAc (A) or of Fru-1,6-P_2 (B), and a tendency was observed for them to be completely released at infinite concentrations of these activators. From the two saturation curves obtained in the presence of L-aspartate, $S_{0.5}$ values for CoASAc and Fru-1,6-P_2 were estimated to be 0.59 and 8.6 mM, respectively, and Hill coefficients (n) to be 2.1 and 1.6, respectively. These results again meet the third criterion. It is interesting that the heterotropic interaction between the inhibitor and activator, which was not observed in the studies using chemical modification, was observed by the ANS method as well as by the kinetic method [24].

Discussion

The maximum number of ANS molecules capable of binding with phospho*enol*pyruvate carboxylase was found to be about 2 per molecule of enzyme subunit, and it was inferred that the two ANS molecules bind with the enzyme with an equal affinity. This permitted a characterization of conformational changes of the enzyme in terms of two paramets, K_f and I_{max}, which were obtained by fluorimetric titration with ANS. The change in fluorescence properties of the enzyme · ANS system caused by single and combined additions of the three kinds of allosteric effectors (L-aspartate, CoASAc, and Fru-1.6-P_2) were shown to reflect closely conformational changes of the enzyme due to allosteric interaction as judged from the three criteria mentioned in Introduction. As seen from Table II, the fluorescence intensity ($I_{max,\infty}$) attainable at infinite concentration of the allosteric inhibitor, L-aspartate, was about 60% of that in its absence, though the dissociation constant (K_f) of the ANS ·

enzyme complex was not affected by L-aspartate. These results indicate that the conformational state induced by L-aspartate was different from the unliganded state. Of the activators, CoASAc caused an increase of $I_{max,\infty}$ by about 30% and an increase of K_f by about 56%, whereas Fru-1,6-P_2 had no effect on both parameters. The possibility that Fru-1,6-P_2 was unable to interact with the enzyme under the experimental conditions employed seemed unlikely from the following two observations: (a) the decrease in I_{max} caused by L-aspartate was released by Fru-1,6-P_2 to the original value under the same conditions (cf. Fig. 7); (b) Previously it was observed that heat-stability of the enzyme was increased by the addition of Fru-1,6-P_2 [11]. It could be said that the conformational states induced by CoASAc and by Fru-1,6-P_2 are different from each other in spite of their similar stimulative effect on the catalytic activity. However, the state induced by Fru-1,6-P_2 could not be distinguished from the unliganded state by this method. The most notable finding in this study was that $I_{max,\infty}$ was decreased by about 30% by combined addition of CoASAc and Fru-1,6-P_2. As mentioned above, neither CoASAc nor Fru-1,6-P_2 caused a decrease in I_{max} when added singly. This indicates unambiguously that the conformational state induced by simultaneous binding of CoASAc and Fru-1,6-P_2 was quite different from either state induced by single binding. Accordingly, it could be said in the present study that four conformational states were discriminated by detection of conformational change through variation at sites other than the functional sites. These four conformational states are depicted in Fig. 8 in which distinct states are denoted as distinct geometrical figures.

The results were essentially in accordance with those obtained by Teraoka et al. [11] in our laboratory using chemical modification of sulfhydryl group(s) at catalytic site with *N*-ethylmaleimide. They showed that at least seven conformational states were accessible to the enzyme upon binding with four kinds of effectors in various combinations. Accordingly, the present result not only gave strong support to the multiplicity of conformational states but also seemed to provide a further information on the nature of conformational changes: the conformational changes were not limited to the area around the

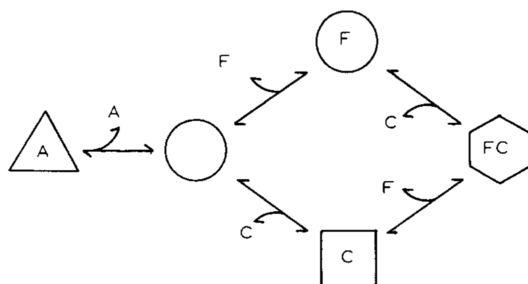

Fig. 8. Proposed model for the multiple conformational states of the enzyme elicited by the allosteric effectors. Geometrical figures (triangle, circle, square, etc.) indicate the respective conformational states of the enzyme, and for simplicity, indication of subunit structure is deleted. Thus, it follows that the states represented by the same figure are not distinguishable in this study. The capital letters denote: A, L-aspartate; F, Fru-1,6-P_2 ; and C, CoASAc.

catalytic site but reached the ANS-binding site(s) which were presumably apart from both catalytic and regulatory sites. Furthermore, another conformational state than those elicited by the individual effectors was shown to be elicited upon simultaneous binding with CoASAc and Fru-1,6-P_2. This phenomenon can be more favorably interpreted in terms of the model of Koshland et al. [25] but not in terms of the two-state model of Monod et al. [26] or of that of Rubin and Changeux [27]. In recent literature similar observations were reported on cytosine triphosphate synthetase (EC 6.3.4.2) [28] and phosphorylase *b* (EC 2.4.1.1) [29]. In these instances an additional conformational state was shown to be induced upon combined addition of the substrate and the effector. However, to my knowledge, no report had appeared which described an additional conformational state induced by cooperation of two kinds of effectors.

Analysis of the conformational state elicited by the third allosteric activator, laurate, could not be carried out in the present investigation. Fluorescence of the enzyme · ANS complex was quenched remarkable by addition of laurate, showing a tendency to complete quenching with increasing concentrations of laurate. This phenomenon might be attributable either to replacement of ANS by laurate at the binding site or to deprivation of the enzyme of ANS by micelles of laurate. From the observations that 7—8 mol of laurate are capable of binding with 1 mol of the enzyme subunit (Izui, K., Yoshinaga, T., Morikawa, M. and Katsuki, H., unpublished work), laurate seems to bind with the enzyme not only at the regulatory site but also at another hydrophobic site for which ANS also has considerable affinity.

Acknowledgment

I would like to express my sincere thanks to Professor H. Katsuki and Dr. K. Izui (Kyoto University) for their encouragement and useful discussions throughout this work.

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