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γ-GLUTAMYL TRANSPEPTIDASE OF KIDNEY BEAN FRUIT

II. STUDIES ON THE ACTIVATING EFFECT OF SODIUM CITRATE

MOSHE Y. GOORE* AND JOHN F. THOMPSON

Section of Biochemistry, Division of Biological Sciences, Cornell University and U.S. Plant, Soil and Nutrition Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ithaca, N.Y. (U.S.A.)

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SUMMARY

1. The effect of sodium citrate and salts of other carboxylic acids on the activation of bean γ-glutamyl transpeptidase has been studied.

2. The possibility that association of subunits or dissociation to subunits occurred in the presence of sodium citrate has been eliminated by the finding that the presence of citrate does not affect the sedimentation behavior of the enzyme.

3. The absence of any effect of urea or guanidine on the activity of the bean transpeptidase indicates that the citrate effect is not due to hydrogen bond breaking.

4. Increase of fluorescence of 1-anilino-8-naphthyl sulfonate in the presence of the transpeptidase when citrate was added indicates that citrate promotes a conformational change favorable for catalytic action.

INTRODUCTION

In the course of investigations on the properties and function of γ-glutamyl transpeptidase from kidney bean fruit, it was observed that upon the addition of sodium citrate to the reaction mixture the enzymic activity was markedly increased[1]. Rather high concentrations (1–1.5 M) of citrate were needed to produce this effect, which is not confined to sodium citrate alone, but is also shown by salts of some dicarboxylic acids and EDTA.

Previous investigations have shown that enhancement of enzymic activity by citrate is due to removal of cations[2], or dissociation of enzyme into subunits[3,4], whereas the effect of citrate on gelatin structure is thought to be a result of breaking hydrogen bonds between protein and bound water[5,6]. Prior work on the bean transpeptidase established that citrate was not inactivating an inhibitor ion[1]. In this paper,

* Present address: Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

it is demonstrated that activation by citrate is a result of a conformational change not primarily caused by hydrogen bond breaking.

MATERIALS AND METHODS

Preparation of the enzyme as well as methods used for the assay are presented in the preceding paper[7].

Ultracentrifugation was carried out in a Spinco Model E centrifuge. A 2.0% solution of purified enzyme in 0.1 M phosphate buffer (pH 6.8) was subjected to centrifugation at 50 470 rev./min (radius at top of cell = 5.7 cm) at 20°.

Fluorescence measurements were carried out in an Aminco–Bowman spectrofluorimeter. Wavelength of exciting light was 310 mμ and fluorescence was measured at 470 mμ. The blank consisted of a solution of the dye in which the protein was omitted.

TABLE I

EFFECT OF SODIUM CITRATE AND SODIUM SALTS OF SOME CARBOXYLIC ACIDS ON HYDROLYTIC ACTIVITY OF BEAN TRANSPEPTIDASE AT pH VALUES OF 6.5 AND 9.5 AND ON TRANSFER ACTIVITY AT pH 9.5

Assay conditions as in preceding paper[7].

Acid	*Final concentration (M)*	*Relative hydrolytic activity**		*Relative transfer activity**
		(pH 6.5)	*(pH 9.5)*	*(pH 9.5)*
None	—	0.58	0.58	0.42
Citric	0.8	1.00	1.00	1.00
Citric	1.2	1.14	1.13	1.16
Oxalic	0.8	0.75	0.80	0.69
Malonic	0.8	0.71	0.70	0.71
Succinic	0.8	0.91	0.89	1.00
Maleic	0.8	0.83	0.62	0.74
Fumaric	0.8	0.78	1.05	0.82
Phthalic	0.8	0.63	0.65	0.51
Acetic	0.8	0.60	0.59	0.45
EDTA	1.2	—	1.04	1.14

* In each group of experiments, the activity at 0.8 M citrate is set at 1.0.

RESULTS AND DISCUSSION

The activating effect of sodium citrate and sodium salts of some other carboxylic acids are illustrated by data in Table I. Inspection of the table also shows that both the hydrolytic and the transfer reactions (see previous paper) are activated to the same extent. Since citrate did not remove interfering cations[1] several other explanations for this effect have been considered:

1. Sodium citrate influences the state of aggregation of the enzyme molecule either by association of subunits or dissociation into subunits[3,4].

2. Sodium citrate interferes with hydrogen bonding, thus loosening and opening the enzyme molecule in a manner similar to that of urea or guanidine.

3. Sodium citrate promotes a stable change in the tertiary structure.

The effect of citrate on the aggregation state of the transpeptidase

In order to determine whether sodium citrate affects the aggregation of the bean transpeptidase as in the case of acetyl CoA carboxylase[2,3], the viscosity and sedimentation behavior of highly purified transpeptidase[7] were determined in the presence and absence of sodium citrate. The viscosity measurements were unsuccessful because the high viscosity of a concentrated sodium citrate solution vitiated any accurate determination of the intrinsic viscosity due to the protein. Measurements of sedimentation velocity were more successful (Fig. 1). Although the transpeptidase was observed as a shoulder on a diffuse peak in sodium citrate solution (Fig. 1), the sedimentation coefficient ($s_{20,w}$) could be evaluated as 4.15, a value close to that of 4.82 obtained in the absence of sodium citrate (Fig. 1).

Accurate measurement of the sedimentation coefficient (Fig. 1) necessitated the use of a relatively high concentration of protein (2.0%) that was about one hundred times as high as the concentration routinely used in activity measurements. Since dissociation of a protein into subunits is generally an equilibrium reaction, the lack of evidence (Fig. 1) for dissociation at the high protein concentrations used in the ultra-

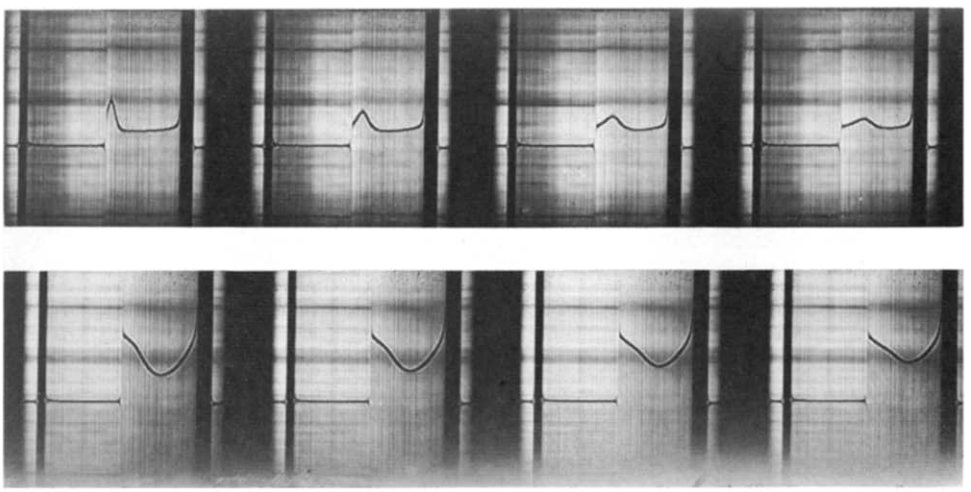

Fig. 1. Sedimentation diagram of kidney bean transpeptidase. Top: sedimentation in the absence of sodium citrate. Photographs taken at 4-min intervals. Bottom: sedimentation in the presence of 1.0 M sodium citrate. Photographs taken at 8-min intervals.

centrifuge may not be pertinent to the conditions under which enzyme assays were normally done. Consequently, the effect of citrate on the activity of the transpeptidase was measured at the concentration of enzyme used in the sedimentation analysis with a correspondingly shorter incubation time. With all other conditions the same as in the standard assays, it was found that 1 M citrate increased transfer activity 2.42-fold and hydrolytic activity 2.39-fold. These results demonstrate that citrate still exerts an activation effect at the protein concentration used in the ultracentrifuge. Therefore, the absence of a citrate effect on the sedimentation coefficient shows that dissociation or association of the enzyme molecule by citrate is not the cause of activation.

TABLE II

THE EFFECT OF UREA AND GUANIDINE ON THE ACTIVITY OF BEAN TRANSPEPTIDASE, AS MEASURED BY THE RELEASE OF *p*-NITROANILINE

Except for the use of the γ-glutamyl-*p*-nitroaniline assay conditions are as described in the previous paper[7].

Compound added	*Final concentration (M)*	*p-nitroaniline released (μmoles)*
None	—	0.19
Sodium citrate	1.5	0.80
Guanidine	0.2	0.16
Guanidine	1.5	0.12
Urea	0.2	0.12
Urea	1.5	0.11

The effect of citrate on hydrogen bonding in transpeptidase

Urea, guanidine and other highly polar substances change protein configuration by breaking hydrogen bonds[8]. The possibility that citrate might activate the bean transpeptidase by breaking hydrogen bonds has been examined by comparing the effects of citrate, urea and guanidine on the activity of the bean transpeptidase. Since urea and guanidine interfere with the assay of transpeptidase with γ-glutamylaniline, γ-glutamyl-*p*-nitroaniline was used as substrate[9]. The results of these experiments, illustrated in Table II, show that neither urea nor guanidine increases the activity of the enzyme. Moreover, when urea and sodium citrate are present together, urea does not reduce the activation by sodium citrate (Table III). It is clear, therefore, that sodium citrate does not enhance the enzyme activity by breaking hydrogen bonds in a manner similar to that of urea or guanidine.

The effect of citrate on conformation of transpeptidase

The possibility that sodium citrate might have some effect on the tertiary structure of the enzyme was also tested by optical rotatory dispersion and absorption of fluorescent dyes. Attempts to determine the effect of citrate on the optical rotatory dispersion of the enzyme were unsuccessful, due to the interference by citrate at a wavelength of 230 mμ where a negative Cotton effect occurred. However, by studying

TABLE III

THE EFFECT OF UREA ON THE ACTIVITY OF BEAN TRANSPEPTIDASE IN THE PRESENCE AND ABSENCE OF SODIUM CITRATE, AS MEASURED BY THE RELEASE OF *p*-NITROANILINE

Assay conditions as in preceding paper[7].

Urea concentration (M)	*p-nitroaniline released in the presence of:*	
	1.5 M sodium citrate (μmoles)	*No extra salts (μmoles)*
0.0	0.685	0.150
0.5	0.685	0.156
2.0	0.456	0.114
4.0	0.410	0.114
8.0	0.296	0.068

TABLE IV

THE EFFECT OF SODIUM CITRATE AND SOME DICARBOXYLIC ACIDS ON THE FLUORESCENCE INTENSITY OF 1-ANILINO-8-NAPHTHYL-SULFONATE IN THE PRESENCE OF γ-GLUTAMYL TRANSPEPTIDASE PREPARATION FROM KIDNEY BEAN FRUIT*

Compound	*Concentration (M)*	*% Fluorescence***
Sodium citrate	0	8.8
Sodium citrate	0.25	55.5
Sodium citrate	0.50	64.5
Sodium citrate	0.75	85
Sodium citrate	1.00	100
Sodium citrate	1.50	100
Succinate	1.00	97
Malonate	1.00	85
Methylcitrate	0.50	5.8

* In each experiment, 2.5 mg/ml protein were used.
** Fluorescence at 1.0 M sodium citrate was set at 100%.

the fluorescence effect of 1-anilino-8-naphthyl sulfonate which becomes fluorescent when it is adsorbed on a protein[10–12], some evidence was obtained that the activation effect is due to conformational changes in the protein molecule. In Table IV, the effect of several salts on the fluorescence intensity of the dye is shown. The methyl ester of citric acid showed neither activation nor the fluorescent effect, thus making it appear that free carboxyl groups are essential for the activation process. Since the activating effect of acetic acid is very low (Table I), it seems that an activating molecule must

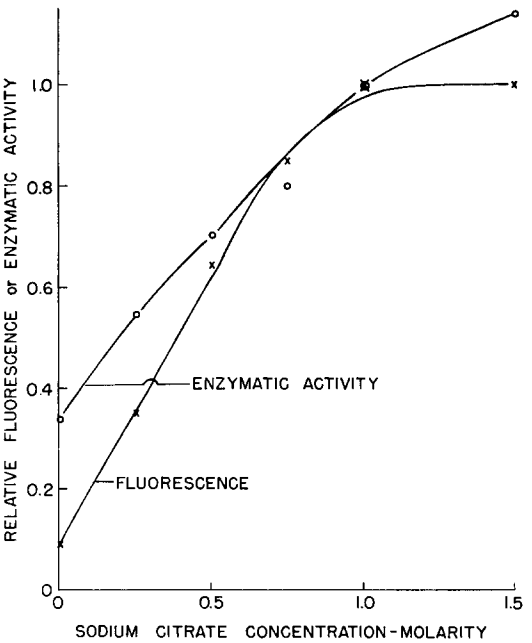

Fig. 2. Effect of sodium citrate on the fluorescence intensity (a) of 1-anilino-8-naphthyl sulfonate in the presence of γ-glutamyl transpeptidase preparation and on the activity (b) of the enzyme. Assay conditions are described in previous paper and fluorescence measurements are described in Table IV.

have at least two carboxyl groups. The steric arrangement of the carboxyl groups appears not to be too important, though a flexible molecule like succinate seems to activate the enzyme more than fumarate (Table I).

The data illustrated in Fig. 2 indicate a close correlation between the effect of citrate on the activity of the enzyme and its effect on the intensity of fluorescence exhibited by the enzyme protein. These results suggest that the effect of sodium citrate on the enzymic activity is due to a change in conformation of the protein molecule. This is in contrast to the effect of citrate on acetyl-CoA carboxylase where activation of the enzyme by citrate is associated with a change in the sedimentation behavior of the enzyme[3,4].

Any physiological significance of the effect of citrate on bean transpeptidase is dubious since the intracellular concentration of this acid is probably much lower[13] than those needed for the activation process of the enzyme.

The effect of citrate on hydrogen bonding in gelatin[5,6] does not appear to be an adequate explanation of the citrate effect on the bean transpeptidase. First, the activation of transpeptidase requires relatively high concentrations of citrate (1.0–1.5 M), whereas helicity in gelatin was promoted by 0.15 M citrate. Secondly, unless citrate acts very specifically on protein–water hydrogen bonds rather than protein–protein hydrogen bonds, it is difficult to understand why urea at low and high concentrations (Tables II and III) does not increase enzyme activity whereas citrate does. Finally, it would be expected that helix formation proposed by COURT AND LITTLE[6] would "tighten" a protein molecule. The previous observation[1] that bromocresol green inhibits the bean transpeptidase only in the presence of citrate is not consistent with a tightening of the protein molecule.

Thus it is concluded that citrate activates the bean transpeptidase by changing the conformation of the protein molecule in such a way that the active site(s) of the enzyme is more accessible to substrates and inhibitors. Specifically, a change in arrangement of highly ordered sections of the molecule without changes within the helical sections can be postulated.

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