THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 244, No. 22, Issue of November 25, pp. 6177-6181, 1969
Printed in U.S.A.

Fructose 1,6-Diphosphatase from Rabbit Liver

XII. EFFECT OF SUBSTRATE AND ADENOSINE MONOPHOSPHATE ON THE IONIZATION OF THE TYROSYL RESIDUES*

(Received for publication, May 26, 1969)

S. PONTREMOLI, E. GRAZI, AND A. ACCORSI
From the Istituto di Chimica Biologica dell'Università di Ferrara, Ferrara, Italy

SUMMARY

The effects of substrate and AMP on the state of ionization of tyrosyl residues of fructose 1,6-diphosphatase have been investigated. In the native enzyme, 5 to 6 tyrosyl residues were titrated with a pK of about 8.4, and 7 to 8 residues were titrated with a pK of 9 to 9.2. The remaining tyrosyl residues were not titrated below pH 10.

In the presence of 10^{-5} M fructose 1,6-diphosphate, when 4 moles of substrate are bound per mole of enzyme, approximately 4 of the low pK tyrosyl residues are shifted from pK 8.4 to pK 9.7. However, all of the pK values are modified, and under these conditions 2 tyrosyl residues show a pK of 8.7, and 10 or 11 residues show a pK of 9.7. At a high concentration of fructose 1,6-diphosphate, 10^{-3} M, all 12 to 13 of the tyrosyl residues are titrated with a pK of 9.8. The results can be correlated with previous studies on the acetylation of fructose 1,6-diphosphatase. The tyrosyl residues with low pK are more reactive and are associated with loss of sensitivity to AMP. However, after acetylation, only 10 tyrosyl residues are titrated with a pK of 9.3 to 9.4, and the pK is shifted to 9.7 when the substrate is bound.

A method of analyzing the titration data is presented which permits simultaneous evaluation of number of residues and pK value of each titrated group. This has revealed changes in tyrosyl residues that would otherwise have been overlooked.

In previous papers (1, 2), we have shown that the catalytic activity and the sensitivity to AMP of fructose 1,6-diphosphatase were altered when the enzyme was treated with reagents, such as diazobenzene sulfonic acid or acetylimidazole. The effects of the above reagents were related to the modification of tyrosyl residues of the enzyme.

The results obtained were consistent with the presence of tyrosyl residues in regions of the enzyme where the substrate or AMP was bound, or, alternatively, in regions of the enzyme modified by the binding of the substrate or of the inhibitor.

* This study was supported by a grant from the Italian Consiglio Nazionale delle Ricerche, Impresa di Enzimologia, and by Grant GM 12291-05 from the National Institutes of Health.

We have now investigated the dissociation of the tyrosyl residues of the enzyme as a function of the pH, by following the spectral changes at 295 mμ in the presence of the substrate and of AMP, and we report the results of our studies in the present paper.

MATERIALS AND METHODS

Fructose 1,6-diphosphatase, specific activity, 120 units per mg of protein, was prepared following the procedure of Pontremoli *et al.* (3), and the specific activity was determined as previously described (4).

The derivatives A and B of fructose 1,6-diphosphatase were prepared by acetylation with acetylimidazole following the procedure already reported (1). Protein concentration was determined with the Folin reagent (5) standardized with a known dry weight of fructose 1,6-diphosphatase.

The ionization of tyrosyl residues of fructose 1,6-diphosphatase was measured from the increase with the pH in the absorbance at 295 mμ *versus* a blank cell containing no enzyme; a molar absorbance coefficient of 2300 for ionized tyrosine was applied (6). The buffers employed in these experiments were the following: 10 mM Tris-HCl between pH 7.5 and 9.0; 10 mM sodium phosphate between pH 6.5 and 7.0; 10 mM diethanolamine-HCl between pH 9.5 and 10.5. Differences in ionic strength due to differences in buffer composition were minimized by the addition of 0.1 M tetraethylammonium chloride. Determinations were made at 20°. The tyrosyl titration curves were analyzed by plotting the hydrogen ion concentration [H+] (*abscissa*) *versus* the reciprocal of the number of the ionized tyrosyl residues, 1/Tyr-O− (*ordinate*). When this plot is used for a set of n tyrosyl residues with the same pK, a straight line is obtained described by the equation

$$[H^+] = nK 1/\text{Tyr-O}^- - K$$

where K is the dissociation constant of the phenolic hydroxyl group of the set of tyrosyl residues considered.

If K is the dissociation constant of the phenolic hydroxyl groups of tyrosyl residues

$$\frac{[H^+][\text{Tyr-O}^-]}{[\text{Tyr-OH}]} = K$$

and n is the total number of tyrosyl residues, dissociated and undissociated

This is an Open Access article under the CC BY license.

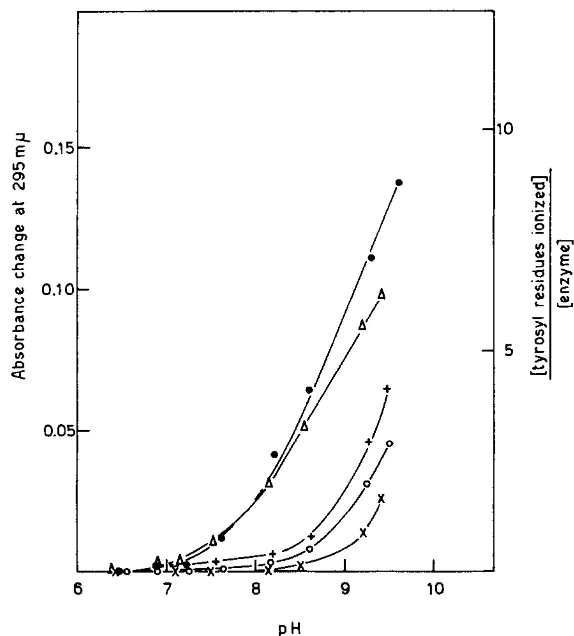

FIG. 1. The effect of the substrate and AMP concentrations on the ionization of tyrosyl residues of fructose 1,6-diphosphatase. *Left ordinate:* increase in the absorbance at 295 mμ over the value at pH 6.5; *right ordinate:* number of tyrosyl residues ionized per molecule of enzyme on the basis of a molar absorbance of 2300 (light path, 1 cm). Enzyme was 6.8 mμmoles; final volume was 1 ml. The buffers employed are described under "Materials and Methods." ●——●, no additions; +——+, 80 mμmoles of fructose 1,6-diphosphate added; ○——○, 1000 mμmoles of fructose 1,6-diphosphate added; △——△, 150 mμmoles of AMP added; ×——×, 150 mμmoles of AMP plus 80 mμmoles of fructose 1,6-diphosphate added.

$$[\text{Tyr-O}^-] + [\text{Tyr-OH}] = n$$

it follows that

$$\frac{[\text{H}^+][\text{Tyr-O}^-]}{[n - \text{Tyr-O}^-]} = K$$

and

$$[\text{H}^+] = nK1/\text{Tyr-O}^- - K$$

The straight line cuts the *ordinate* at a point giving $1/n$ and it cuts the *abscissa* at a point which gives $-K$. If two sets of tyrosyl residues with different pK are considered, a curve represented by two straight lines with different slopes is obtained, and both the dissociation constant and the number of the two sets of tyrosyl residues can be determined graphically.

RESULTS

Effects of pH, Substrate, and AMP on Absorption at 295 mμ of Fructose 1,6-Diphosphatase—The titration curve of the tyrosyl residues of fructose 1,6-diphosphatase is altered by the presence of the substrate, fructose 1,6-diphosphate. In fact, the ionization of the phenolic hydroxyl groups, as revealed by the increase in the absorbance at 295 mμ, is strongly depressed in the pK range tested, pH 7.5 to 10, when the substrate is added to the native enzyme (Fig. 1). This effect on the ionization of tyrosyl residues is already detected at a substrate concentration of the order of 0.1 μM (Table I, Fig. 2). The association constant for the binding of the substrate to the enzyme can be estimated from

TABLE I

Effect of substrate concentration on binding of substrate and on ionization of tyrosyl residues of fructose 1,6-diphosphatase

Fructose diphosphatase	Fructose 1,6-diphosphate added	Free fructose 1,6-diphosphate concentration (calculated)	Substrate bound per mole of enzyme (calculated)	Increase in the absorbance at 295 mμ	Tyrosine residues ionized per molecule of enzyme
mμmoles		*μM*	*moles*		
6.7	0	0.00	0.00	0.112	7.27
6.7	5	0.05	0.80	0.092	5.97
6.7	10	0.12	1.49	0.082	5.32
6.7	20	0.50	2.85	0.072	4.67
6.7	30	4.37	3.83	0.056	3.63
6.7	40	13.6	3.94	0.047	3.05
6.7	60	33.3	3.98	0.043	2.78
6.7	80	53.3	4.00	0.043	2.78
6.7	100	73.3	4.00	0.037	2.53
6.7	1000	973	4.00	0.030	1.94

The titration was performed at pH 9.2 in the presence of 10 mM Tris-HCl containing 0.1 M tetraethylammonium chloride. Final volume was 1 ml. Free fructose 1,6-diphosphate concentration was calculated from the association constant of 0.5×10^7 M^{-1} for the reaction of formation of the enzyme-(substrate)$_4$ complex (4) by making use of the total enzyme concentration and of the total amount of substrate added. By a similar procedure, the number of moles of substrate bound per mole of enzyme was calculated. The number of tyrosyl residues ionized per molecule of enzyme was calculated from the increase in the absorbance at 295 mμ over the value at pH 6.5, in the absence of the substrate, and on the basis of a molar absorbance of 2300.

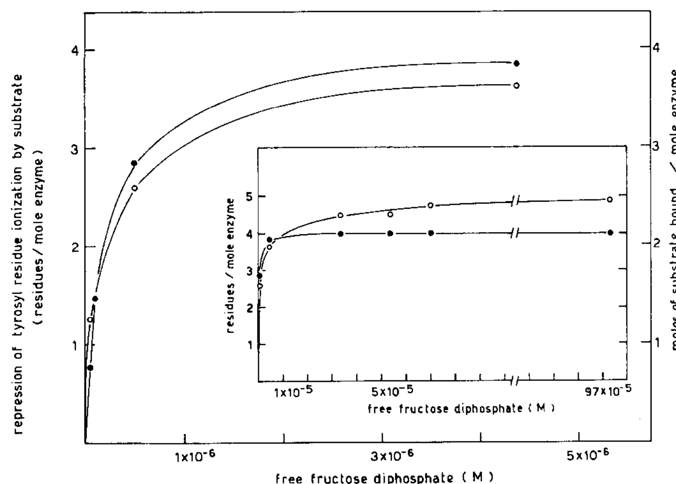

FIG. 2. Effect of substrate concentration on the binding of the substrate and on the ionization of tyrosyl residues of fructose 1,6-diphosphatase at pH 9.2. Data are taken from Table I. ●——●, moles of substrate bound per mole of enzyme (calculated from the association constant of 0.5×10^7 M^{-1} for the reaction of formation of the enzyme-(substrate)$_4$ complex (4)); ○——○, repression of tyrosyl residues ionization by substrate (residues per mole of enzyme).

the spectral modification to be 1.5 to 1×10^7 M^{-1}, a value which is in agreement with that of 0.5×10^7 M^{-1} calculated from the direct measurement of the binding of the radioactive substrate to the enzyme at pH 9.2 (4).

From an analysis of the titration curves of Fig. 1, it is possible to estimate the pK values and the number of the tyrosyl residues

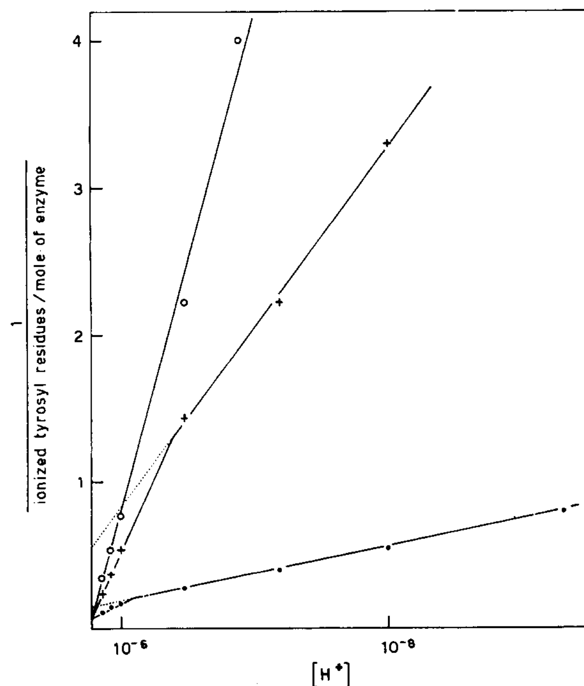

FIG. 3. Plot of the reciprocal of the number of tyrosyl residues ionized per molecule of enzyme *versus* the hydrogen ion concentration. Data are taken from Fig. 1. ●——●, native enzyme; +——+, native enzyme plus 80 mμmoles of fructose 1,6-diphosphate; ○——○, native enzyme plus 1000 mμmoles of fructose 1,6-diphosphate.

titrated under the various conditions (Fig. 3). In the absence of substrate, the tyrosyl residues titratable between pK 7.5 and 10 can be divided into two groups: a first group of 5 or 6 tyrosyl residues with a pK of 8.4, and a second group of 7 or 8 tyrosyl residues with a pK of 9 to 9.2.

In the presence of 53 μM fructose 1,6-diphosphate, when approximately 4 moles of substrate are bound per mole of enzyme (4), the ionization of 4 tyrosyl residues appears to be completely repressed (Figs. 1 and 2). However, analysis of the titration curve (Fig. 3) shows that the effect of the substrate is more complicated and is related to a change in the ionization of a larger set of tyrosyl residues. The low pK group is now restricted to 2 tyrosines and the pK of the high pK group has been increased. No hydrolysis of the substrate occurs in the absence of the cation.

At higher substrate concentration (973 μM), the effect on the spectra is larger (Figs. 1 to 3). The group of tyrosyl residues with low pK is no longer detectable, and a further small increase in the pK of the tyrosyl residues of the second group has occurred. Substrate effects are summarized in Table II.

Effect of Some Phosphoric Esters on Absorption at 295 mμ of Fructose 1,6-Diphosphatase—The effect of some phosphoric esters on the dissociation of the tyrosyl residues of fructose 1,6-diphosphatase has also been studied. Fructose 6-phosphate, glucose 6-phosphate, and fructose 1-phosphate show, on the tyrosyl titration curve, an effect similar to that of fructose 1,6-diphosphate but which is detectable only at concentrations larger than 0.1 mM. The simultaneous addition of fructose 1,6-diphosphate and fructose 6-phosphate decreases the absorption at 295 mμ to the same level as does a saturating concentration of fructose 1,6-diphosphate alone (Table III). Galactose 6-phosphate, D-gluconate 6-phosphate, sorbitol 1,6-diphosphate, and mannitol 1,6-diphosphate at 1 mM concentration do not modify the spectrum. Among the sugar phosphates tested, fructose 1-phosphate occupies a particular position. It is a poor substrate of fructose 1,6-diphosphatase, at pH 7.5 it is hydrolyzed at 2 to 3% of the rate of fructose 1,6-diphosphate, and, as is clear from Table III,

TABLE II

Effect of substrate on ionization of tyrosyl residues of fructose 1,6-diphosphatase

	Low pK group		High pK group	
	No.	pK	No.	pK
Native enzyme	5–6	8.4	7–8	9–9.2
+ 53 μM fructose 1,6-diphosphate	1.7	8.7	10–11	9.7
+ 973 μM fructose 1,6-diphosphate	0		13	9.8
Acetylated enzyme (Derivative A)			10	9.3–9.4
+ 53 μM fructose 1,6-diphosphate			10	9.7
+ 973 μM fructose 1,6-diphosphate			10	10

TABLE III

Effects of some phosphoric esters on absorption at 295 mμ of fructose 1,6-diphosphatase

The mixtures (final volume, 1 ml) contained 5.7 mμmoles of fructose 1,6-diphosphatase and the compound tested as indicated. The buffers employed were as indicated under "Materials and Methods." The increase in the absorbance was measured over the value at pH 6.5.

Addition	Increase in the absorbance at 295 mμ	
	pH 8.4	pH 9.4
None	0.038	0.085
D-Fructose 1,6-diphosphate		
0.1 μmole	0.006	0.049
1.0 μmole	0.002	0.027
D-Fructose 6-phosphate		
0.1 μmole	0.022	0.079
1.0 μmole	0.001	0.044
D-Fructose 1,6-diphosphate + D-fructose 6-phosphate		
1 μmole each		0.028
D-Glucose 6-phosphate		
0.1 μmole	0.036	0.085
1.0 μmole	0.026	0.066
D-Fructose 1-phosphate		
0.1 μmole	0.039	
1.0 μmole	0.034	
4.0 μmoles	0.027	
D-Galactose 6-phosphate		
1.0 μmole	0.037	
D-Gluconate 6-phosphate		
1.0 μmole	0.037	
D-Sorbitol 1,6-diphosphate + D-mannitol 1,6-diphosphate		
1.0 μmole	0.039	

TABLE IV

Effect of acetylation of fructose 1,6-diphosphatase on binding of substrate and of AMP

Specific activity was determined at pH 9.2 as previously described (4). The inhibition by AMP of the hydrolysis of 1 mM fructose 1,6-diphosphate was evaluated at 2° in the presence of 1 mM $MgCl_2$ and of 0.02 M Tris-acetate buffer, pH 7.5. Orthophosphate was determined by the method of Fiske and SubbaRow (9). The binding of the radioactive substrate and of AMP was determined at 2° and at pH 7.5 as previously described (4, 7).

	Specific activity	Inhibition by AMP		Fructose 1,6-diphosphate		AMP	
		0.1 mM	1 mM	10 μM	100 μM	4.5 μM	100 μM
		%		*moles bound/mole enzyme*			
Native enzyme	120	95	98	3.9	4.0	0.9	4.1
Derivative A	90	20	48	2.8	3.1	0.4	1.8
Derivative B	0			0.0	0.0		

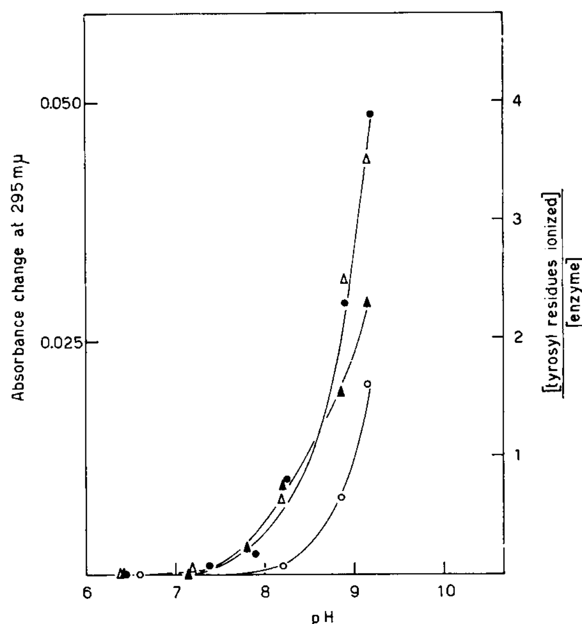

FIG. 4. The effect of fructose 1,6-diphosphate and AMP on the tyrosyl titration curve of acetylated phosphatase (Derivative A). *Left ordinate:* increase in absorbance at 295 mμ over the value at pH 6.5; *right ordinate:* number of tyrosyl residues ionized per molecule of enzyme on the basis of a molar absorbance of 2300 (light path, 1 cm). Enzyme was 5.5 mμmoles, final volume was 1 ml. The buffers employed are described under "Materials and Methods." ●——●, acetylated enzyme; ○——○, acetylated enzyme plus 1 μmole of fructose 1,6-diphosphate; △——△, acetylated enzyme plus 0.1 μmole, or ▲——▲, 1 μmole of AMP.

it exhibits an affinity for the enzyme which is lower than that of glucose 6-phosphate and fructose 6-phosphate.

Effect of AMP on Absorption at 295 mμ of Fructose 1,6-Diphosphatase—In addition to the substrate, AMP also represses the ionization of the tyrosyl residues of fructose 1,6-diphosphatase. Under the conditions of the experiment represented in Fig. 1, approximately 4 moles of AMP are bound per mole of enzyme (7); nevertheless, the spectral change is much less pronounced than when 4 moles of substrate are bound. Since both the substrate and the inhibitor repress the dissociation of tyrosyl residues, the question arises whether the same residues are involved in the phenomenon. To answer this question, a titration experiment was performed under conditions where 4 moles of substrate and 4 moles of the inhibitor were bound per mole of enzyme. As shown in Fig. 1, the effects of fructose 1,6-diphosphate and of AMP on the ionization of tyrosyl residues were additive. Since at the pH and at the concentrations used we have no evidence of mutual influences of either the substrate or the inhibitor on the binding to the enzyme (8), we can conclude that different tyrosyl residues are responsible for the effects of the above compounds on the titration curve.

Tyrosyl Titration Curve of Acetylated Fructose 1,6-Diphosphatase—We have previously reported that acetylation with acetylimidazole of fructose diphosphatase alters both the catalytic and the allosteric properties of the protein (1). Acetylation of 6 tyrosyl residues (Derivative A) desensitizes the enzyme to the inhibition by AMP; acetylation of 10 to 12 tyrosyl residues (Derivative B) abolishes the catalytic activity.

We have further investigated the properties of these two enzyme derivatives by studying the binding of the radioactive substrate and inhibitor with the Sephadex G-50 filtration technique (Table IV) (4, 7) and the effects of the above compounds on the tyrosyl titration curve (Fig. 4, Table II). Derivative B, which is completely inactive, has also lost completely the capability to bind the substrate. Derivative A is 75% active and binds fructose 1,6-diphosphate at 10 μM practically with the same affinity as does the native enzyme, whereas it binds AMP at 0.1 mM to only a fraction of the original extent, which is related to the residual sensitivity to the inhibitor. The tyrosyl titration curve of Derivative A is distinctly different from that of the native enzyme. Analysis of the tyrosyl titration curve with the procedure described in Fig. 3 reveals that the acetylated residues are those with the lowest pK (pK 8.4). The spectrum of Derivative A is, however, still sensitive to the addition of the substrate (Fig. 4), the effect being related to the increase from 9.3 and 9.4 to 10 of the pK of 10 tyrosyl residues.

The effect of 0.1 mM AMP on the spectrum is, on the contrary, no longer detectable, although with 1 mM AMP a small effect is still observed, together with a small increase in inhibition of fructose 1,6-diphosphate hydrolysis.

DISCUSSION

Out of a total number of 38 tyrosyl residues (10), fructose 1,6-diphosphatase possesses 13 to 14 residues, titratable between pH 7.5 and 10, which can be differentiated into two groups, a first group of 5 to 6 residues (pK 8.4) and a second group of 7 to 8 residues (pK 9 to 9.2).

The ionization of such residues is decreased by the binding of the substrate and, to a smaller extent, by the binding of AMP. More specifically, the tyrosyl residues titration curve is influenced by the binding of fructose 1,6-diphosphate to the same sites which have been detected by direct measurement in the presence of the radioactive substrate (4). This conclusion is supported by the fact that at pH 9.2 the same association constant can be calculated either from the direct binding measurements or from the spectral changes. This latter phenomenon would appear to involve the complete repression of the dissociation of 4 tyrosyl residues under conditions (fructose 1,6-diphosphate, 53 μM) in which 4 moles of substrate are bound per mole of enzyme. However, analysis of the data reveals that a larger number of tyrosyl residues contributes to the total spectral change, and that

3 to 4 tyrosyl residues of the group with low pK are shifted to the group with high pK, while the pK of the latter group is increased.

The spectral modification becomes even more pronounced when the substrate concentration is increased to 973 μM. Additional substrate binding sites with an association constant of the order of 10^6 M^{-1} must thus be present in addition to the 4 binding sites detected at low substrate concentration (association constant, 1 to 1.5×10^7 M^{-1}).

This is in keeping with the effects on substrate such as substrate inhibition (11) and the cooperative effect on the binding of AMP (7), which occur only at concentrations higher than 0.1 mM.

Spectral modifications as a function of the concentrations of hydrogen ion, of substrate, and of AMP have also been studied on Derivative A, which was obtained by acetylation of fructose 1,6-diphosphatase with acetylimidazole. These studies have shown that acetylimidazole attacks preferentially the tyrosyl residues with the lowest pK. The effect of the acetylation of these residues on the affinity for the substrate and on the rate of the reaction is, however, negligible. The low pK tyrosyl residues do not seem, therefore, to be important for the catalytic activity. These groups, on the contrary, affect the reaction with AMP. Both the binding and the inhibition in the presence of 0.1 mM AMP are decreased in comparison with the native enzyme, and the spectral change is abolished. The inhibition and the spectral change, however, are partially restored with 1 mM AMP.

Both our previous results on acetylation of fructose 1,6-diphosphatase (1) and the present results indicate the existence in fructose 1,6-diphosphatase of two distinct regions, one responsible for the binding of AMP and the other responsible for the binding of the substrate. Furthermore, it is also clear that these regions mutually influence each other. This is shown in acetylation studies by the fact that desensitization to AMP and inactivation are prevented by the presence, respectively, of AMP and of fructose 1,6-diphosphate, as well as, although to a minor extent, by the binding of the other effector to the allosteric region: fructose 1,6-diphosphate for the desensitization to AMP and AMP for the inactivation. In the titration experiments, the same phenomenon is also clearly detected. Fructose 1,6-diphosphate, in fact, extends its influence also to the low pK tyrosyl residues which are in the region for the AMP binding and which are the first to be affected by acetylation. In addition, the acetylation of the tyrosyl residues of the AMP region alters the pK of the high pK tyrosyl residues which are presumably in the substrate region. It is therefore clear from our studies that the AMP and the substrate binding regions mutually interact and that their interaction can be followed through the changes in the ionization of the two groups of tyrosyl residues that have been detected.

REFERENCES

1. Pontremoli, S., Grazi, E., and Accorsi, A., *Biochemistry*, **5**, 3072, 3568 (1966).
2. Pontremoli, S., Grazi, E., and Accorsi, A., *J. Biol. Chem.*, **242**, 61 (1967).
3. Pontremoli, S., Traniello, S., Luppis, B., and Wood, W. A., *J. Biol. Chem.*, **240**, 3459 (1965).
4. Pontremoli, S., Grazi, E., and Accorsi, A., *Biochemistry*, **7**, 1655 (1968).
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
6. Crammer, J. L., and Neuberger, A., *Biochem. J.*, **37**, 302 (1943).
7. Pontremoli, S., Grazi, E., and Accorsi, A., *Biochemistry*, **7**, 3628 (1968).
8. Pontremoli, S., Grazi, E., and Accorsi, A., *Biochem. Biophys. Res. Commun.*, **33**, 335 (1968).
9. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
10. Pontremoli, S., Luppis, B., Traniello, S., Rippa, M., and Horecker, B. L., *Arch. Biochem. Biophys.*, **112**, 7 (1965).
11. Taketa, K., and Pogell, B. M., *J. Biol. Chem.*, **240**, 651 (1965).