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Essential Arginyl Residues in Fructose-1,6-bisphosphatase†

Frank Marcus

ABSTRACT: Modification of pig kidney fructose-1,6-bisphosphatase with 2,3-butanedione in borate buffer (pH 7.8) leads to the loss of the activation of the enzyme by monovalent cations, as well as to the loss of allosteric adenosine 5′-monophosphate (AMP) inhibition. In agreement with the results obtained for the butanedione modification of arginyl residues in other enzymes, the effects of modification can be reversed upon removal of excess butanedione and borate. Significant protection to the loss of K^+ activation was afforded by the presence of the substrate fructose 1,6-bisphosphate, whereas AMP preferentially protected against the loss of AMP inhibition. The combination of both fructose 1,6-bisphosphate and AMP fully protected against the changes in enzyme properties on butanedione treatment. Under the latter conditions, one arginyl residue per mole of enzyme subunit was modified, whereas three arginyl residues were modified by butanedione under conditions leading to the loss of both potassium activation and AMP inhibition. Thus, the modification of two arginyl residues per subunit would appear to be responsible for the change in enzyme properties. The present results, as well as those of a previous report on the subject (Marcus, F. (1975), *Biochemistry 14,* 3916-3921) support the conclusion that one arginyl residue per subunit is essential for monovalent cation activation, and another arginyl residue is essential for AMP inhibition. A likely role of the latter residue could be its involvement in the binding of the phosphate group of AMP.

Mammalian liver and kidney fructose-1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) are tetrameric enzymes composed of four presumably identical subunits with molecular weights of approximately 35 000 (Mendicino et al., 1972; Tashima et al., 1972; Traniello et al., 1972). Each enzyme subunit possesses at neutral pH a single substrate binding site (Pontremoli et al., 1968a; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974), and a divalent metal ion binding site (Pontremoli et al., 1969). The enzyme is also activated by monovalent cations (Hubert et al., 1970). In addition, an allosteric site for the highly specific inhibitor AMP[1] is also present per enzyme subunit (Pontremoli et al., 1968b; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974). Based on recent structural studies (Abrams et al., 1975), liver and kidney fructose-1,6-bisphosphatase appear to have similar primary structures.

From chemical modification studies it has been established that arginyl residues play an essential role in many enzymes by participating in the recognition of anionic ligands. Examples include the participation of enzyme arginyl residues at the binding sites of phosphorylated substrates (Daemen and Riordan, 1974; Borders and Riordan, 1975; Lobb et al., 1975; Powers and Riordan, 1975), pyridine nucleotides (Yang and Schwert, 1972; Lange et al., 1974; Bleile et al., 1975; Blumenthal and Smith, 1975), and carboxylic substrates (Riordan, 1973; Riordan and Scandurra, 1975; Werber et al., 1975). With this background, I initiated a study on the modification of pig kidney fructose-1,6-bisphosphatase with the arginine specific reagent, 2,3-butanedione. The existence of positively charged recognition sites on the enzyme appeared a priori likely, since both the substrate fructose 1,6-bisphosphate and the allosteric inhibitor AMP, are negatively charged. Indeed, an ϵ-amino group of lysine has already been identified at the C-6 phosphate binding site of the substrate by modification studies of pig kidney fructose-1,6-bisphosphatase with pyridoxal-P (Colombo and Marcus, 1974). Unexpectedly, the first results of modification of fructose-1,6-bisphosphatase with butanedione led to the recognition of highly reactive arginyl residues that are essential for the monovalent cation activation of the enzyme (Marcus, 1975). However, it was also noticed that significant losses of AMP inhibition can also occur upon modification. The latter finding prompted a continuation of the study of the modification of fructose-1,6-bisphosphatase by butanedione. The experiments reported herein demonstrate that arginyl residues are also essential for AMP inhibition of fructose-1,6-bisphosphatase.

Materials and Methods

Pig kidney fructose-1,6-bisphosphatase with optimal activity at neutral pH was purified as previously described (Colombo and Marcus, 1973). Its protein concentration was determined spectrophotometrically at 280 nm using the extinction coefficient $E_{1cm}^{1\%}$, of 7.55 (Marcus and Hubert, 1968). Based on a subunit molecular weight of 35 000 (Mendicino et al., 1972), the molecular weight of the enzyme tetramer was taken as 140 000 for all calculations. Rabbit liver fructose-1,6-bisphosphatase was partially purified as previously described (Marcus, 1975).

Fructose-1,6-bisphosphatase activity was measured as described (Marcus, 1975). The assays were carried out at 30 °C

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[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; NADP, nicotinamide adenine dinucleotide phosphate.

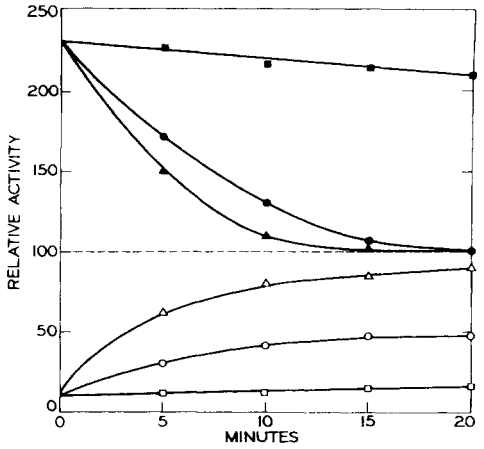

FIGURE 1: Time course of changes of kidney fructose-1,6-bisphosphatase activity on modification with butanedione. Enzyme (2.8 μM) was reacted at 30 °C with 7 mM butanedione in a reaction system containing 60 mM borate buffer (pH 7.8), 0.2 mM $MgSO_4$, and 0.3 mM EDTA, under three different conditions: (▲, △) no other additions, (●, ○) plus 0.6 mM AMP, (■, □) plus 0.6 mM AMP and 1.3 mM fructose-1,6-bisphosphate. At the indicated times, aliquots were removed, diluted 20-fold in cold 100 mM borate buffer (pH 7.8) containing 0.1 mM EDTA, and assayed at 30 °C for enzyme activity. The filled symbols indicate assays in the presence of 150 mM K^+, and the empty symbols indicate assays in the presence of 67 μM AMP. A relative value of 100 is given to the specific activity (13.6) of the control enzyme assayed in the absence of K^+, and this activity remained constant in all modification conditions (broken line).

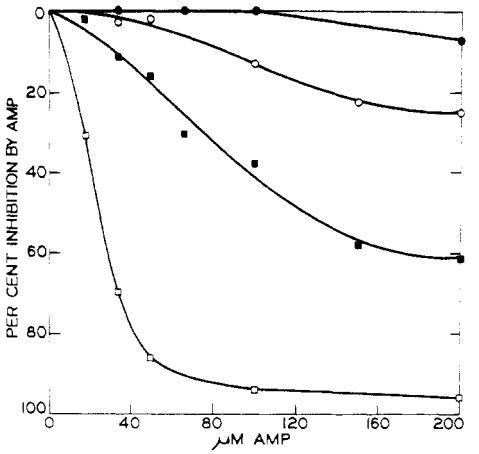

FIGURE 2: AMP inhibition of control and butanedione-modified fructose-1,6-bisphosphatases. Pig kidney fructose-1,6-bisphosphatase (2.8 μM) was reacted at 30 °C with 7 mM butanedione in a reaction system containing 60 mM borate buffer (pH 7.8), 0.2 mM $MgSO_4$, and 0.3 mM EDTA. At various time intervals, aliquots were removed, diluted 20-fold in cold 100 mM borate buffer (pH 7.8) containing 0.1 mM EDTA, and assayed. The control enzyme was subjected to the same treatments, except for the omission of butanedione. Enzyme assays were performed in the absence of K^+ as indicated under Materials and Methods, except that AMP was added as indicated. (□) control enzyme; (■, ○, ●) modified 5, 10, and 20 min, respectively.

either in the presence or absence of 150 mM K^+ (75 mM K_2SO_4) in an assay system that contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-NaOH buffer (pH 7.5), 0.17 mM fructose 1,6-bisphosphate (Sigma stock 750-1), 5 mM $MgSO_4$, 0.1 mM EDTA, 0.3 mM NADP, phosphoglucose isomerase (2.3 units/ml), and glucose-6-P dehydrogenase (0.6 unit/ml). The reaction was initiated by the addition of fructose-1,6-bisphosphatase. Except for an initial lag period of about 2 min in assays performed in the presence of 150 mM K^+, reaction rates were constant.

Modification of fructose-1,6-bisphosphatase with 2,3-butanedione (Sigma) was carried out at 30 °C as described (Marcus, 1975). The reaction system contained 2.8 μM fructose-1,6-bisphosphatase, 7 mM butanedione, 60 mM borate buffer (pH 7.8), 0.2 mM $MgSO_4$, and 0.3 mM EDTA. Other additions are indicated in the legends to figures. At various time intervals aliquots were removed, diluted in cold 100 mM borate buffer (pH 7.8) containing 0.1 mM EDTA, and assayed for enzyme activity. Whenever required, aliquots were also removed and added to 0.4 volume of 6 N HCl to stop the modification reaction and to prevent the regeneration of free arginine (Riordan, 1973). The modification of arginyl residues was then determined by the loss of arginine upon amino acid analysis (Marcus, 1975).

In some experiments, modified enzyme was separated at 23 °C from the other reaction components by passage through a Sephadex G-100 column (1.8 × 28 cm) equilibrated in different buffers, as mentioned in the text. This procedure was useful not only for the separation of the enzyme from excess reagent, but also to check that the modified enzyme was not dissociated into subunits. For the latter purpose the effluent from the column was connected to an ultraviolet absorption meter (Gilson Medical Electronics UV 2801), and percent transmittance at 280 nm was continuously monitored on an Esterline Angus model AW recorder. The resolution of the system proved to be satisfactory, as tested with samples containing variable proportions of pig kidney fructose-1,6-bisphosphatase (mol wt 140 000) and two × crystallized bovine hemoglobin (molecular weight 64 500).

Results

Modification of Pig Kidney Fructose-1,6-bisphosphatase with Butanedione. Incubation of fructose-1,6-bisphosphatase with butanedione can result in pronounced effects on the properties of the enzyme. Depending on the modification conditions, the activation of the enzyme by monovalent cations and the allosteric inhibition of fructose-1,6-bisphosphatase by AMP can be affected. In a previous paper (Marcus, 1975), high concentrations of AMP were systematically included in the modification reaction system to restrict the modification of arginyl residues presumably involved in AMP inhibition. As a result of these studies, it was concluded that one of two reactive arginyl residues per mole of enzyme subunit was essential for monovalent cation activation. Modification conditions have now been selected to study the loss of AMP inhibition on reaction of the enzyme with butanedione. The time-course of changes in enzyme activity on incubation of pig kidney fructose-1,6-bisphosphatase with 7 mM butanedione in borate buffer (pH 7.8) in the absence of AMP is shown in Figure 1, triangles. As expected, a progressive loss of the activation of the enzyme by potassium ions was observed, although the activity of the enzyme in the absence of added potassium remained constant (Figure 1, broken line). Under these experimental conditions the decrease in potassium activation obeyed pseudo-first-order kinetics with a rate constant of 0.25 min^{-1}. However, concomitant with the loss of potassium activation, there was also a progressive loss of inhibition of fructose-1,6-bisphosphatase by 67 μM AMP. A more detailed analysis of the loss of AMP inhibition as a function of the modification time (Figure 2) showed that after 5 min of modification the K_i for AMP had already increased to 125 μM from its initial value of 20 μM. The AMP inhibition continued to decrease with longer modification times, and after 20 min of modification the modified enzyme exhibited nearly no inhibition by 200 μM AMP, a concentration equal to ten times

TABLE I: Reversibility of the Butanedione Modification of Fructose-1,6-bisphosphatase.[a]

	Treatment	Act. Ratio $+K^+/-K^+$	% AMP Inhibition
(1)	None	2.49	88
(2)	Butanedione	1.18	2
(3)	Butanedione + gel filtration in Tris buffer	2.40	40
(4)	Butanedione + gel filtration in Tris buffer + 0.25 mM AMP	2.35	83
(5)	Butanedione + gel filtration in borate buffer	1.05	3

[a] Pig kidney fructose-1,6-bisphosphatase (2.8 μM) was reacted at 30 °C in a reaction system containing 7 mM butanedione, 60 mM borate buffer (pH 7.8), 0.2 mM $MgSO_4$, and 0.3 mM EDTA. After 15 min, an aliquot was removed for assays (2). Parts of the reaction mixture were subjected to gel filtration on Sephadex G-100 columns (1.8 × 28 cm) equilibrated in different buffers: (3) 20 mM Tris-HCl (pH 7.5), containing 2 mM $MgSO_4$ and 0.1 mM EDTA; (4) 20 mM Tris-HCl (pH 7.5), containing 2 mM $MgSO_4$, 0.1 mM EDTA, and 0.25 mM AMP; (5) 50 mM borate buffer (pH 7.8), containing 0.05 mM EDTA. Assays were performed in the presence, as well as in the absence of 150 mM K^+ as described under Materials and Methods. AMP inhibition was measured at 67 μM AMP in the absence of potassium.

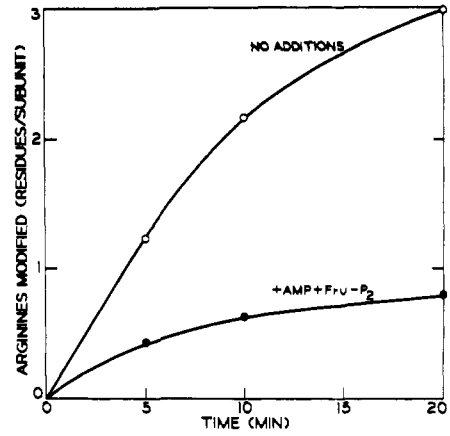

FIGURE 3: Arginine modification of kidney fructose-1,6-bisphosphatase with butanedione. Enzyme (2.8 μM) was reacted at 30 °C with 7 mM butanedione in 60 mM borate buffer (pH 7.8), 0.2 mM $MgSO_4$, 0.3 mM EDTA, in either the presence or absence of 5 mM fructose-1,6-bisphosphate plus 5 mM AMP. Aliquots were removed at the times indicated, the reaction was stopped by the addition of 0.4 volume of 6 N HCl, and samples were processed for amino acid analysis. Results are expressed as the number of modified arginyl residues per subunit, based on the loss of arginine indicated by amino acid analysis. The control enzyme had 10.9 arginines per subunit.

the initial K_i. Some inhibition was observed at higher AMP concentrations (i.e., 1 mM AMP), but this appears to be due to AMP reversal of butanedione modification (see later). Other properties tested with the 20-min-modified enzyme remained nearly identical to those of a control enzyme. These included: pH optimum (7.4), K_a for Mg^{2+} (0.4–0.5 mM), K_m for fructose 1,6-bisphosphate (lower than 10 μM), and inhibition by high substrate concentrations.

Preferential protection to the effects of butanedione modification was obtained by the presence of either the substrate fructose 1,6-bisphosphate or the inhibitor AMP in the modification system. Fructose 1,6-bisphosphate (1.3 mM) selectively protected against loss of monovalent cation activation by butanedione treatment, but afforded nearly no protection against the loss of AMP inhibition. Conversely, the presence of 0.6 mM AMP exerted no protection against the loss of monovalent cation activation but partially protected against the loss of AMP inhibition (Figure 1, circles). These results suggest the existence of at least two different sites of reaction of butanedione with fructose-1,6-bisphosphatase. Also shown in Figure 1 are the results of the modification of fructose-1,6-bisphosphatase in the presence of both fructose 1,6-bisphosphate and AMP, conditions which resulted in full protection from the effects of butanedione modification (Figure 1, squares).

Reversibility of the Effects of Butanedione. In agreement with the results obtained for the butanedione modification of arginyl residues in other enzymes (Riordan, 1973; Daemen and Riordan, 1974; Bleile et al., 1975; Borders and Riordan, 1975), the effects of modification of fructose-1,6-bisphosphatase by butanedione were reversed upon removal of excess butanedione and borate. This was demonstrated by reacting fructose-1,6-bisphosphatase with butanedione for 15 min, which resulted in a nearly complete loss of both potassium activation and AMP inhibition (Table I, expt 2). Butanedione and borate were then removed by gel filtration through a Sephadex G-100 column, equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgSO_4$, and 0.1 mM EDTA. This treatment (Table I, expt 3) resulted in full restoration of the potassium activation, and in a partial restoration of AMP inhibition. However, AMP inhibition was also fully restored when the above buffer also contained 0.25 mM AMP (Table I, expt 4). Neither potassium activation nor AMP inhibition was restored if gel filtration was performed in borate buffer (Table I, expt 5). In all cases, the activity of the enzyme measured in the absence of K^+ remained constant. The enzyme fraction always emerged in the void volume of the Sephadex G-100 column, indicating that no dissociation of the modified enzyme into subunits occurred on modification.

Modification of Arginyl Residues. To correlate the changes in enzyme properties with arginine modification, the basic amino acid composition of samples of fructose-1,6-bisphosphatase modified with butanedione was analyzed. Two extreme conditions were chosen for modification. Thus, samples were modified in either the absence or presence of both 5 mM fructose 1,6-bisphosphate and 5 mM AMP. The time course of arginine modification of these two samples is shown in Figure 3. The results demonstrate modification in the absence of fructose 1,6-bisphosphate and AMP (conditions leading to the loss of AMP inhibition, as well as of potassium activation; cf. Figure 1), three arginyl residues per mole of enzyme subunit were modified in 20 min. Neither lysyl nor histidyl residues were modified with butanedione. Modification of enzyme protected by the presence of 5 mM fructose 1,6-bisphosphate plus 5 mM AMP reduced the loss of arginyl residues to 0.8 in 20 min. Within this modification period no change in enzyme properties was observed. Hence, it appears that the modification of approximately two arginyl residues per mole of enzyme subunit accounted for the loss of both monovalent cation activation and AMP inhibition of fructose-1,6-bisphosphatase. The results obtained in shorter modification times (Figure 3) are also consistent with the above proposal. For instance, after 10 min of modification a differential loss of 1.55 arginyl residues per mole of enzyme subunit had occurred, and the modified enzyme was already nearly insensitive to both potassium activation and AMP inhibition (Figure 1). Since it has been

previously shown that only one arginyl residue per subunit is essential for monovalent cation activation (Marcus, 1975), it would appear that the other one plays an essential role in allosteric AMP inhibition of fructose-1,6-bisphosphatase.

Modification of Rabbit Liver Frustose-1,6-bisphosphatase by Butanedione. To test further the relevance of the results obtained by modification of pig kidney fructose-1,6-bisphosphatase with butanedione, a few modification experiments were also performed with partially purified rabbit liver fructose-1,6-bisphosphatase. The results of these experiments were all consistent with those obtained with the pig kidney enzyme. Under conditions identical to those described in the legend to Figure 1, the incubation of partially purified rabbit liver fructose-1,6-bisphosphatase (0.53 mg/ml; specific activity, 1.6) with 7 mM butanedione for 30 min resulted in the complete loss of both potassium activation and AMP inhibition. The presence of 5 mM fructose 1,6-bisphosphate in the modification system protected against loss of potassium activation, but afforded no protection against the loss of AMP inhibition. Conversely, the presence of 5 mM AMP protected against the loss of AMP inhibition, but exerted no protection against the loss of monovalent cation activation.

Discussion

The chemical modification of several enzymes with 2,3-butanedione in borate buffers has demonstrated that this reagent is a powerful tool for establishing the functional role of arginyl residues (Yang and Schwert, 1972; Riordan, 1973; Daemen and Riordan, 1974; Lange et al., 1974; Bleile et al., 1975; Riordan and Scandurra, 1975; Marcus et al., 1976). In most cases, reaction occurs with only a small number of the total arginyl residues. The reason for this selectivity is not yet known, but it has been suggested that the reactive arginyl residues may be located in an hydrophobic environment that enhances their reactivity (Powers and Riordan, 1975).

From the results presented herein, and a previous report on the subject (Marcus, 1975), it is clear that arginyl residues also play an essential role in fructose-1,6-bisphosphatase. Indeed, two properties of fructose-1,6-bisphosphatase, monovalent cation activation and AMP inhibition, can be lost by reaction of enzyme arginyl residues with butanedione (Figure 1, triangles). However, modification with butanedione does not appear to result in structural changes of the active site region, since enzyme activity measured in the absence of potassium, as well as other properties related to the active site, remained unchanged after modification. Protection experiments indicate that two distinct regions appear to be involved in potassium activation and in AMP inhibition. Modification in the presence of fructose 1,6-bisphosphate selectively protects against the loss of monovalent cation activation, and this protective effect appears to be due to an enzyme conformational change promoted by high substrate concentrations (Marcus, 1975). Conversely, modification in the presence of AMP (Figure 1) results in significant protection against the loss of AMP inhibition but affords nearly no protection against the loss of potassium activation. Since the affinity of the enzyme for AMP is increased in the presence of substrate (Kratowich and Mendicino, 1974), modification in the presence of substrate plus inhibitor (Figure 1) increases the protective effect of AMP against the loss of AMP inhibition. The experiments on the reversibility of the butanedione modification (Table I) could also be taken as a confirmation for the existence of these two distinct regions, since full recovery of potassium activation could be obtained with only a partial reversal of the loss of AMP inhibition (Table I, expt. 3). This could mean that the arginyl residues involved in potassium activation are more exposed to the solvent than those involved in AMP inhibition. The fact that the presence of AMP favors reversal of the loss of AMP inhibition (cf. expt. 3 and 4, Table I) could well be due to a displacement of the equilibrium of the reaction Enz-Arg·butanedione (complex I, in the scheme proposed by Riordan, 1973) $\rightleftharpoons$ Enz-Arg + butanedione, in favor of the formation of a fructose-1,6-bisphosphatase-AMP complex (Enz-Arg + AMP $\rightleftharpoons$ Enz-Arg·AMP). This would be expected if the modification has occurred at the AMP binding site.

From the results of amino acid analysis of the enzyme modified under various experimental conditions (Table I, Marcus (1975); Figure 3, this report), it would appear that one reactive arginyl residue per subunit has neither a role in enzyme activity nor in AMP inhibition, since these are not altered by modification of the enzyme in the presence of high concentrations of fructose-1,6-bisphosphate plus AMP. A second reactive arginyl residue is essential for monovalent cation activation (Marcus, 1975), and a third reactive arginyl residue per subunit of fructose-1,6-bisphosphatase is essential for AMP inhibition. Although other interpretations are possible (i.e., indirect disruption of the AMP site; requirement of the modified residue in a conformational change induced by AMP), this arginyl residue could well be part of the allosteric site of fructose-1,6-bisphosphatase to bind the negatively charged phosphate group of AMP. The participation of an arginyl residue for the binding of ligands containing the AMP moiety has been proposed in several other examples of enzyme modification studies. These include dehydrogenases (Lange et al., 1974; Bleile et al., 1975), kinases (Borders and Riordan, 1975; Berghauser, 1975), glutamine synthetase and carbamyl phosphate synthetase (Powers and Riordan, 1975), and mitochondrial ATPase (Marcus et al., 1976). The participation of an arginyl residue could well be a general feature of AMP binding sites. It is already known from the crystallographic structure of horse liver alcohol dehydrogenase-AMP complex (Zeppezauer et al., 1975) that the residue that seems to be mainly responsible for binding the phosphate group of AMP is the guanidinium group of Arg-47 that projects into an otherwise hydrophobic region. Although the essential arginyl residue of horse liver alcohol dehydrogenase that is modified by butanedione has not been identified in the primary structure (Lange et al., 1974), there are strong indications favoring Arg-47 as the site of reaction (Lange et al., 1975).

Desensitization of fructose-1,6-bisphosphatase from several sources to AMP inhibition has also been obtained by chemical modification of tyrosyl (Rosen and Rosen, 1966; Pontremoli et al., 1966), and lysyl residues (Rosen and Rosen, 1966; Marcus, 1967; Marcus and Hubert, 1968; Krulwich et al., 1969; Colombo et al., 1972). Although these reports did not establish that the modification occurred in residues at or close to the AMP binding site, it could also be the case. Indeed, this could be suggested from the results of Pontremoli et al. (1966) showing that the acetylation of four tyrosyl residues per mole of rabbit liver fructose-1,6-bisphosphatase with *N*-acetylimidazole is associated with the loss of allosteric AMP inhibition, and those of Colombo et al. (1972) demonstrating that the modification of four lysyl residues per mole of pig kidney fructose-1,6-bisphosphatase with pyridoxal-P results also in loss of AMP inhibition. The possibility of physical proximity of the arginyl residue essential for AMP inhibition with the above mentioned tyrosyl and lysyl residues merits further investigations.

Acknowledgments

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