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# Functional Consequences of Modifying Highly Reactive Arginyl Residues of Fructose 1,6-Bisphosphatase. Loss of Monovalent Cation Activation†

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ABSTRACT: Modification of pig kidney fructose 1,6-bisphosphatase with 2,3-butanedione (in the presence of AMP) results in the loss of activation of the enzyme by monovalent cations. Under these conditions about 8 arginyl residues per mole of enzyme were modified. No other residues were modified. No loss of monovalent cation activation occurs when modification with 2,3-butanedione is carried out in the presence of AMP plus the substrate fructose 1,6-bisphosphate and 3.2 less arginyl residues were modified. Since fructose 1,6-bisphosphatase contains 4 subunits, it is suggested that one arginyl residue per subunit plays an essential role in monovalent cation activation of the enzyme. Studies on sulfhydryl group reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid) explain the protection exerted by fructose 1,6-bisphosphate against the loss of monovalent cation activation in terms of an enzyme conformational change induced by substrate, which makes unreactive the essential arginyl residue. The results of the present paper, as well as previous evidence, are discussed in terms of the mechanism of monovalent cation activation of fructose 1,6-bisphosphatase.

Fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase), the enzyme that catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, is a regulatory enzyme that plays a key role in the control of gluconeogenesis (for a review see Pontremoli and Horecker, 1971). The native forms of liver and kidney fructose 1,6-bisphosphatase exhibit maximum activity at neutral pH (Traniello et al., 1971, 1972; Tashima et al., 1972; Colombo and Marcus, 1973), require $Mg^{2+}$ or $Mn^{2+}$ for activity, and are allosterically inhibited by AMP. The enzyme is 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). At neutral pH it possesses four binding sites for the substrate (Pontremoli et al., 1968a; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974), four divalent metal ion binding sites (Pontremoli et al., 1969), as well as four allosteric sites for the inhibitor AMP per tetrameric enzyme molecule (Pontremoli et al., 1968b; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974).

Fructose 1,6-bisphosphatase is also activated by monovalent cations, as first mentioned by Hers and Eggermont (1964), and more extensively studied by Hubert et al. (1970). The latter report demonstrated that the enzyme from various vertebrate sources was activated by monovalent cations, potassium or ammonium being the best activators. In addition, it was also shown that the presence of the monovalent cation activator altered other properties of fructose 1,6-bisphosphatases (i.e., AMP inhibition, Mg-saturation curves).

With only a few exceptions (Behrisch, 1971; Black et al., 1972; Gonzalez et al., 1972; Hochachka, 1972; Villanueva and Marcus, 1974), the effect of monovalent cations on fructose 1,6-bisphosphatase has been ignored by most workers in the field, perhaps due to the fact that this property of fructose 1,6-bisphosphatases is one of the properties of the enzyme which is lost upon proteolytic conversion of neutral to alkaline fructose 1,6-bisphosphatase (Colombo and Marcus, 1973; Gonzalez et al., 1974). The present report demonstrates that the monovalent cation activation of fructose 1,6-bisphosphatase can also be abolished by chemical modification of highly reactive arginyl residues of the enzyme with 2,3-butanedione, an arginine-specific reagent which has been successfully used in the past few years for the recognition of the role of arginyl residues in several enzymes (Huang and Tang, 1972; Yang and Schwert, 1972; Riordan, 1973; Daemen and Riordan, 1974; Lange et al., 1974).

## Materials and Methods

Fructose 1,6-bisphosphatase activity was determined spectrophotometrically by following the rate of formation of NADPH at 340 nm in the presence of excess phosphoglu-

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Table I: Basic Amino Acid Composition of Native and Modified Fructose 1,6-Bisphosphatases.[a]

| Enzyme | Act. Ratio $+K^+/-K^+$ | Basic Amino Acids (Residues/mol) Lys | His | Arg | Loss of Arg (Residues/mol)[b] |
|---|---|---|---|---|---|
| 1. Control | 2.3 | 87.1 | 13.6 | 45.7 | |
| 2. Modified in the presence of 4.4 m*M* AMP | 1.0 | 87.0 | 13.8 | 37.5 | 8.2 |
| 3. Modified in the presence of 4.4 m*M* AMP plus 2.1 m*M* Fru-$P_2$ | 2.3 | 89.4 | 14.1 | 40.8 | 4.9 |

[a] Modification of kidney fructose 1,6-bisphosphatase (1.8 $\mu M$) with 7.5 m*M* butanedione was carried out at 30° in 60 m*M* borate buffer (pH 7.8), 60 $\mu M$ EDTA, and other additions as indicated. After 30 min of incubation, aliquots were assayed for activity in the presence, as well as in the absence, of 150 m*M* $K^+$, and others were subjected to amino acid analysis. [b] Difference in arginine residues in the native and modified enzymes.

cose isomerase and glucose-6-phosphate dehydrogenase. The assays were carried out either in the presence or absence of 150 m*M* $K^+$ (75 m*M* $K_2SO_4$) in an assay system of 3 ml which contained 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)–NaOH buffer (pH 7.5), 0.17 m*M* fructose 1,6-bisphosphate (Sigma stock 750-1), 5 m*M* $MgSO_4$, 0.1 m*M* EDTA, 0.3 m*M* NADP, phosphoglucose isomerase (2.3 units/ml), glucose-6-P dehydrogenase (0.6 unit/ml), and fructose 1,6-bisphosphatase. Assays were carried out at 30°. A unit of fructose 1,6-bisphosphatase activity is defined as that amount of enzyme which catalyzes the formation of 1 $\mu$mol of fructose-6-P/min under the conditions described above. Specific activity is expressed in terms of units per milligram of protein.

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 by its absorbancy at 280 nm using a value of 0.755 for absorbancy $mg^{-1}$ $ml^{-1}$ (Marcus and Hubert, 1968). Based on a subunit molecular weight of 35,000 (Mendicino et al., 1972), the molecular weight of the enzyme was taken as 140,000 for all calculations. The specific activity of the purified kidney enzyme was 30.8 units/mg when assayed in the presence of 150 m*M* $K^+$ and 13.4 units/mg in its absence.

Rabbit liver fructose 1,6-bisphosphatase was partially purified from fresh livers by a method which employed the first three steps (extraction, heat fractionation, and acid fractionation) of the procedure of Traniello et al. (1971), followed by ammonium sulfate fractionation (0.30–0.65 saturation), and P-cellulose chromatography as previously described in the preparation of pig kidney fructose 1,6-bisphosphatase (Colombo et al., 1972). The specific activity of this partially purified preparation was 10.1 units/mg when assayed in the presence of 150 m*M* $K^+$, and it was 4.2 units/mg in its absence.

Modification of fructose 1,6-bisphosphatase with 2,3-butanedione (Sigma) was carried out at 30°. The reaction components were incubated for 10 min at 30° before the addition of butanedione. All other conditions are indicated in the figure legends. Solutions of butanedione of a concentration tenfold higher than required for modification were freshly prepared for each experiment in 100 m*M* boric acid–borax buffer (pH 7.8) (Gomori, 1955), containing 0.1 m*M* EDTA. (This buffer solution is referred to in the text as "borate buffer".) Since the pH falls on the addition of butanedione to the borate buffer solution (Riordan, 1973), it was readjusted to pH 7.8 with 1 *N* NaOH before addition to the modification mixture. No further adjustments were required during the 30-min period of modification. Most of the studies of the properties of modified enzyme (referred to in the text as "butanedione-modified fructose 1,6-bisphosphatase") were performed on a sample which was prepared by treating 1.8 $\mu M$ pig kidney fructose 1,6-bisphosphatase with 7.5 m*M* butanedione in a reaction system containing 60 m*M* borate buffer (pH 7.8), 60 $\mu M$ EDTA, and 4.4 m*M* AMP. All components, excepting butanedione, were incubated for 10 min at 30°. Then, butanedione was added and incubation was continued for 30 min at 30°. After this period, the modified enzyme was separated at room temperature from the other reaction components by passage through a Sephadex G-50 (fine) column (1.8 × 28 cm) equilibrated in 50 m*M* borate buffer (pH 7.8), containing 0.1 m*M* EDTA.

Titration of enzyme sulfhydryl groups with $Nbs_2$[1] (Ellman, 1959) was carried out at 23° and the rate of formation of the thionitrobenzoate anion was followed at 412 nm with a Gilford Model 2000 spectrophotometer. Reactions were performed in borate buffer and other additions were as indicated in the figure legends. Reactions were always started by the addition of $Nbs_2$. The absorbance of a blank containing buffer and $Nbs_2$ was continually subtracted from that of the reaction mixture. A molar extinction coefficient of 13,600 for the liberated thionitrobenzoate anion was used for all calculations.

For amino acid analysis, fructose 1,6-bisphosphatase was treated with butanedione as indicated in the legend to Table I and the reaction was stopped by the addition of 0.4 vol of 6 *N* HCl. Addition of acid also prevents the regeneration of free arginine (Riordan, 1973). The precipitated protein was collected by centrifugation, washed several times with 6 *N* HCl, and finally suspended in 6 *N* HCl. Hydrolysis was carried out in sealed, evacuated ampoules at 110° for 24 hr and amino acid analysis was carried out on a Beckman 120C amino acid analyzer using the single-column methodology (Beckman publication no. A-TB-059A, Oct, 1972).

## Results

*Modification of Pig Kidney Fructose 1,6-Bisphosphatase with 2,3-Butanedione.* Initial experiments of modification of pig kidney fructose 1,6-bisphosphatase with 2,3-butanedione showed that treatment with this reagent resulted in significant changes in the properties of the enzyme, affecting both enzyme activity and allosteric AMP inhibition. The observed alteration in properties was highly dependent on the composition of the modification system and it appeared that modification in the presence of the substrate fructose 1,6-bisphosphate resulted in loss of AMP inhibition, while modification in the presence of AMP preferentially led to partial loss of enzyme activity. The latter conditions were more fully explored since the results appeared to follow a pattern rather similar to the modification of essential arginyl residues at the active site of *Escherichia coli* alkaline phosphatase with 2,3-butanedione (Daemen and Riordan, 1974). Figure 1 shows the time course of the loss

[1] Abbreviation used is: $Nbs_2$, 5,5'-dithiobis(2-nitrobenzoic acid).

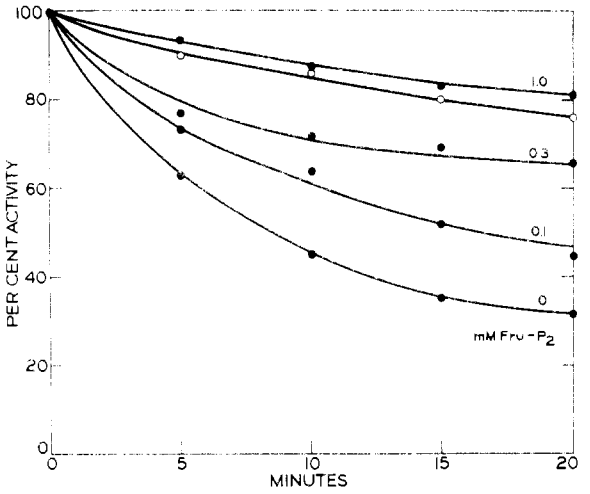


FIGURE 1: Time course of changes of kidney fructose 1,6-bisphosphatase activity on modification with butanedione. Fructose 1,6-bisphosphatase (4 $\mu M$) was allowed to react at 30° with 10 m$M$ butanedione in a reaction system containing 60 m$M$ borate buffer (pH 7.8), 60 $\mu M$ EDTA, 2.2 m$M$ AMP, and either fructose 1,6-bisphosphate at the indicated concentrations (●) or 1 m$M$ fructose-6-P (○). At the times indicated, aliquots were removed, diluted 30-fold in cold 100 m$M$ borate buffer (pH 7.8) containing 0.1 m$M$ EDTA, and assayed at 30° for fructose 1,6-bisphosphatase activity in the presence of 150 m$M$ $K^+$.

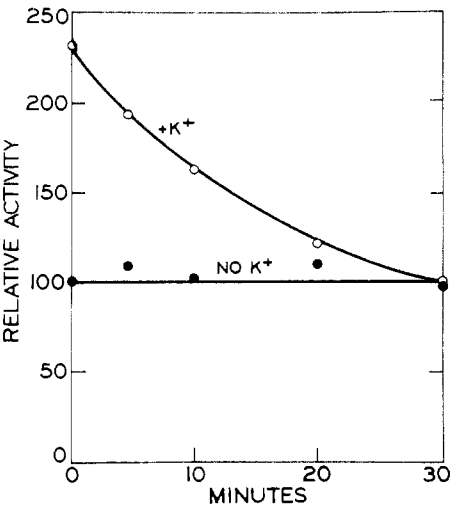


FIGURE 2: Time course of changes of kidney fructose 1,6-bisphosphatase activity on modification with butanedione in the presence of AMP. Enzyme (1.8 $\mu M$) was allowed to react at 30° with 7.5 m$M$ butanedione in a reaction system containing 60 m$M$ borate buffer (pH 7.8), 60 $\mu M$ EDTA, and 4.4 m$M$ AMP. At the indicated times, aliquots were removed, diluted 40-fold in cold 100 m$M$ borate buffer (pH 7.8) containing 0.1 m$M$ EDTA, and assayed at 30° for fructose 1,6-bisphosphatase activity in the presence (○) as well as in the absence (●) of 150 m$M$ $K^+$. A relative activity value of 100 is given to the specific activity of the native enzyme assayed in the absence of $K^+$.

of fructose 1,6-bisphosphatase activity when the enzyme is allowed to react in the presence of 2.2 m$M$ AMP with 10 m$M$ butanedione, as well as the protection against enzyme inactivation afforded by the addition of substrate fructose 1,6-bisphosphate or the product fructose-6-P. Neither 5 m$M$ $MgSO_4$ nor 0.5 $M$ KCl protected the enzyme from inactivation by butanedione. The above results appeared to be at first sight suggestive of modification of the enzyme at or near the active site, except that under no conditions was the inactivation of fructose 1,6-bisphosphatase with 2,3-butanedione greater than 60–65%. Higher concentrations of the reagent (up to 20 m$M$) affected only the rate but not the final extent of inactivation. A closer examination of the problem by following several activity parameters during modification of kidney fructose 1,6-bisphosphatase with butanedione in the presence of AMP demonstrated that the apparent loss of activity was due to loss of monovalent cation activation rather than a true loss of enzymatic activity. This is clearly shown in Figure 2, where activity changes were followed by measurements of fructose 1,6-bisphosphatase activity in either the presence or absence of 150 m$M$ $K^+$, while in the previously described experiments shown in Figure 1, activity was measured only in a routine assay system containing 150 m$M$ $K^+$ as monovalent cation activator. As seen in Figure 2, incubation of fructose 1,6-bisphosphatase with 7.5 m$M$ butanedione in the presence of 4.4 m$M$ AMP resulted in the complete loss of potassium activation after 30 min of incubation, with no change of fructose 1,6-bisphosphatase activity measured in the absence of potassium.

Similar results to those shown in Figure 2 were obtained when rabbit liver fructose 1,6-bisphosphatase (0.53 mg/ml) was allowed to react at 30° with 10 m$M$ butanedione in a reaction system containing 60 m$M$ borate buffer (pH 7.8), 60 $\mu M$ EDTA, and 4.4 m$M$ AMP. After 30 min of treatment, the activation of rabbit liver fructose 1,6-bisphosphatase by 150 m$M$ $K^+$ decreased from its initial value of 2.4-fold to 1.17 with no changes in activity measured in the absence of monovalent cation activator. As already demonstrated for the modification of other enzymes with butanedione in borate buffers (Riordan, 1973; Daemen and Riordan, 1974; Lange et al., 1974), the reaction was highly specific for the modification of arginyl residues, and none of the other amino acids of pig kidney fructose 1,6-bisphosphatase was decreased as indicated by amino acid analysis of acid hydrolysates of butanedione-modified fructose 1,6-bisphosphatases. Table I includes the basic amino acid composition of fructose 1,6-bisphosphatase samples modified under two different conditions (lines 2 and 3) compared to a control enzyme (line 1). Amino acid analysis of fructose 1,6-bisphosphatase treated 30 min in the presence of 4.4 m$M$ AMP with 7.5 m$M$ butanedione (cf. Figure 2) demonstrated the loss of 8.2 arginyl residues per enzyme molecule (Table I, line 2). The presence of 4.4 m$M$ AMP plus 2.1 m$M$ fructose 1,6-bisphosphate in the modification system reduced the loss of arginyl residues to 4.9 (Table I, line 3). Thus, 3.3 arginyl residues per mole of enzyme were not modified due to the presence of a substrate concentration that fully protected from the loss of potassium activation.

*Properties of the Enzyme Modified in the Presence of AMP.* As can be seen by comparison of Figures 3A and 3B, the modified enzyme was no longer activated by monovalent cations. Other properties of fructose 1,6-bisphosphatase (Table II) remain nearly unchanged with the sole exception of the sensitivity of the enzyme to AMP inhibition. $K_i$ for AMP increases more than fourfold due to modification. However, the loss of sensitivity to AMP inhibition and the loss of monovalent cation activation would appear to be separate events, since AMP inhibition can be selectively altered with no loss of monovalent cation activation, if the modification of fructose 1,6-bisphosphatase with 2,3-butanedione is carried out without AMP, but in the presence of fructose 1,6-bisphosphate. Neither sensitivity to AMP inhibition nor activation by monovalent cations is altered when modification is carried out in the presence of both fructose 1,6-bisphosphate and AMP. The modification con-

Table II: Properties of Native- and Butanedione-Modified Fructose 1,6-Bisphosphatase.[a]

| | Native | Modified |
|---|---|---|
| pH optimum | 7.2–7.4 | 7.2–7.4 |
| $K_m$ for Fru-$P_2$ | <10 $\mu M$ | <10 $\mu M$ |
| % inhibition by excess Fru-$P_2$[b] | 41 | 31 |
| $K_a$ for $Mg^{2+}$ | 0.35 m$M$ | 0.41 m$M$ |
| $K_i$ for AMP | 25 $\mu M$ | 112 $\mu M$ |

[a] Modified with 7.5 m$M$ butanedione in the presence of 4.4 m$M$ AMP, as described under Materials and Methods. All assays were carried out in the absence of $K^+$. [b] For substrate inhibition, activity was measured at 1 m$M$ Fru-$P_2$ and a relative value of 100% was given to the activity measured at 0.1 m$M$ substrate.

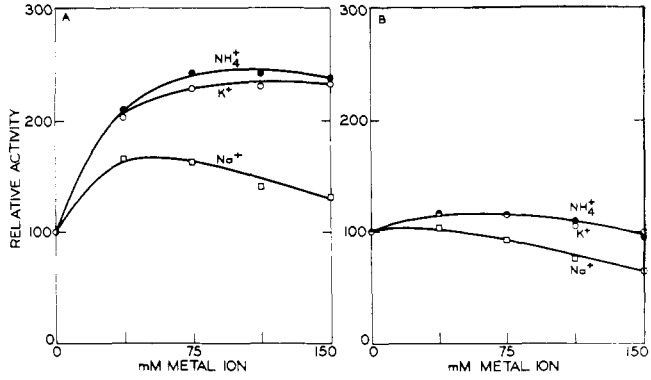


FIGURE 3: Effect of monovalent cations on the activity of native (A) and butanedione-modified (B) kidney fructose 1,6-bisphosphatase. The assays were performed as described under Materials and Methods, except that different monovalent cations (as their sulfate salts) were added as indicated. Butanedione-modified fructose 1,6-bisphosphatase was prepared as described under Materials and Methods.

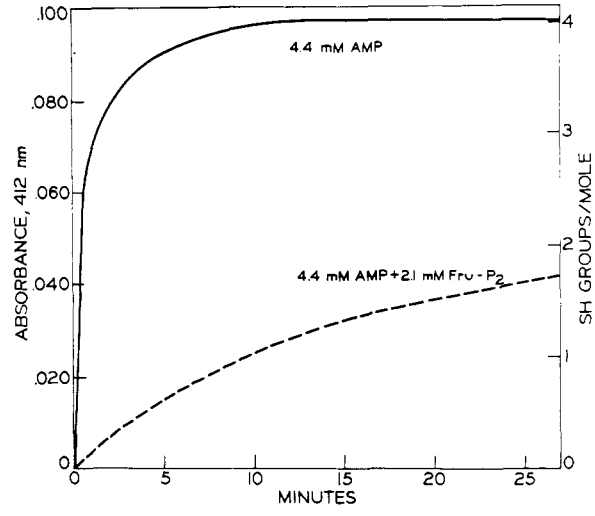


FIGURE 4: Reaction of kidney fructose 1,6-bisphosphatase with $Nbs_2$. Enzyme (1.8 $\mu M$) was allowed to react at 23° with 0.25 m$M$ $Nbs_2$ in a reaction system containing 60 m$M$ borate buffer (pH 7.8), 60 $\mu M$ EDTA, and other additions as indicated below: solid line, 4.4 m$M$ AMP; dashed line, 4.4 m$M$ AMP plus 2.1 m$M$ fructose 1,6-bisphosphate.

ditions (presence of fructose 1,6-bisphosphate) which lead to the selective loss of AMP inhibition are now being studied in more detail and will be the subject of a future communication.

*Sulfhydryl Reactivity as a Measure of Conformational Changes.* Measurements of sulfhydryl group reactivity with $Nbs_2$, as an indicator of conformational changes (Guidotti, 1965; Kemp and Forest, 1968; Kemp, 1969; Rao et al., 1969; Wasserman and Major, 1969), demonstrate that the protection exerted by high concentrations of fructose 1,6-bisphosphate against the loss of monovalent cation activation can be explained in terms of conformational changes of the enzyme. As shown in Figure 4, when the enzyme is allowed to react with $Nbs_2$ in the presence of 4.4 m$M$ AMP (conditions used in Figure 2, which lead to the loss of monovalent cation activation), four SH groups were titrated in the first 10 min, and no further reaction occurred in 120 min. On the other hand, reaction in the presence of 4.4 m$M$ AMP plus 2.1 m$M$ fructose 1,6-bisphosphate (conditions which lead to no change in enzyme properties) shows no fast reacting SH groups, but a very slow reaction (only 2.6 SH groups had been titrated in 120 min) with perhaps another set of SH groups. When either the native or the butanedione-modified enzyme was allowed to react with $Nbs_2$ in the presence of 0.1% sodium dodecyl sulfate the titration was rapid and the number of titrable SH groups was found to be 20 ± 0.3 cysteines/mol of enzyme, in good agreement with amino acid analysis data of pig kidney fructose 1,6-bisphosphatase indicating 22 half-cysteines/mol of enzyme (Mendicino et al., 1972). Values ranging from 16 to 24 cysteines/mol of enzyme have been reported for other kidney, liver, and muscle fructose 1,6-bisphosphatases (Fernando et al., 1969; Byrne et al., 1971; Black et al., 1972; Tashima et al., 1972; Traniello et al., 1972; Traniello, 1974).

Although the mechanism of monovalent cation activation of fructose 1,6-bisphosphatase has not been eludicated yet, previous evidence (see Discussion section) suggests that the enzyme could fit a general mechanism in which monovalent cations exert a role by maintaining or promoting a specific protein conformation necessary for optimum catalytic activity (Evans and Sorger, 1966; Suelter, 1974). If this were the case, measurements of SH reactivity could also provide a tool for demonstrating a conformational effect of monovalent cations on fructose 1,6-bisphosphatase. Figure 5A, which shows a time course of the reaction of the sulfhydryl groups of fructose 1,6-bisphosphatase with $Nbs_2$ in the presence and in the absence of 150 m$M$ $K^+$, indeed demonstrates that a significant change occurs in the presence of $K^+$ since both the rate as well as the extent of reaction of SH groups of fructose 1,6-bisphosphatase are markedly affected. In the absence of $K^+$, approximately three to four SH groups were titrated rapidly (in approximately 15 min), and four additional SH groups were titrated in 120 min. In the presence of $K^+$, reaction with the four fast reacting SH groups still occurred, but apparently the second set of four SH groups was nearly unreactive. Thus, four SH groups per mole of enzyme became either directly blocked by $K^+$, or most likely these groups became unreactive due to a conformational change induced by the presence of 150 m$M$ $K^+$. Measurements of SH group reactivity with the butanedione-modified fructose 1,6-bisphosphatase which had lost the monovalent cation activation (Figure 5B) showed that modification nearly abolished the response of the second set of SH groups to the presence of 150 m$M$ $K^+$, and approximately eight SH groups were titrated in 120 min in both the presence and absence of 150 m$M$ $K^+$. Thus, it appears that modification of a highly reactive arginyl residue of fructose 1,6-bisphosphatase prevents a monovalent cation induced conformational change which results in an enzyme conformation with higher catalytic activity. It should be

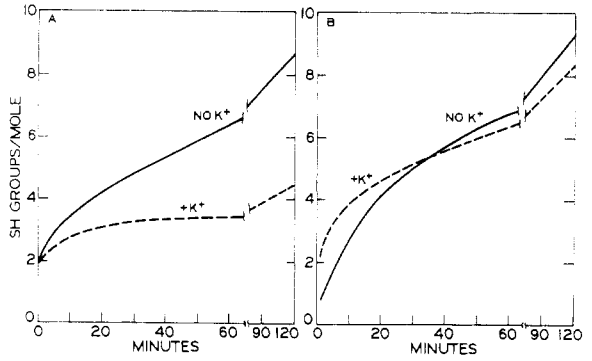


FIGURE 5: Effect of $K^+$ on the reaction of kidney fructose 1,6-bisphosphatase with $Nbs_2$: (A) native enzyme (0.61 $\mu M$) was allowed to react at 23° with 0.15 m$M$ $Nbs_2$ in a reaction system containing 50 m$M$ borate buffer (pH 7.8) and 50 $\mu M$ EDTA, in either the presence (dashed line) or absence (solid line) of 75 m$M$ $K_2SO_4$; (B) as above, but with 0.61 $\mu M$ butanedione-modified fructose 1,6-bisphosphatase.

noted, however, that modification with butanedione appears to alter somewhat the native enzyme conformation as is reflected in a slight decrease in the rate of reaction of the fast reacting SH groups after modification (compare the results shown by the solid lines of Figures 5A and 5B). However, this is not surprising since it is very unlikely that any modification of a protein can be achieved without a slight conformational change (Cohen, 1970).

## Discussion

Although a considerable number of enzymes are activated by monovalent cations (for reviews see Evans and Sorger, 1966; Suelter, 1970, 1974), the mechanisms by which monovalent cations activate certain enzymes are still poorly understood. However, accumulated evidence appears to point out that for many enzymes a common mechanism may be the interaction of the cation at a site distant from the catalytic site to promote an enzyme conformation of higher catalytic activity, rather than the direct participation of the monovalent metal in the reaction mechanism. Thus, monovalent cations would act as positive allosteric effectors affecting the equilibrium between two or more enzyme conformations (Suelter, 1974). In the case of fructose 1,6-bisphosphatases, several previous lines of evidence appear to be in accord with the above interpretation. Among these: (a) in several fructose 1,6-bisphosphatases the allosteric inhibition of the enzyme by AMP is highly dependent on both the concentration and nature of monovalent cations (Hubert et al., 1970; Gonzalez et al., 1972; Black et al., 1972; Hochachka, 1972; Behrisch and Johnson, 1974; Villanueva and Marcus, 1974); (b) Mg-saturation curves are markedly altered in the presence of monovalent cations (Hubert et al., 1970; Black et al., 1972; Hochachka, 1972); (c) differential effects of temperature on monovalent cation activation have been observed (Behrisch, 1971); (d) by proteolytic treatment it is possible to remove monovalent cation activation while retaining enzyme activity (Colombo and Marcus, 1973; Gonzalez et al., 1974). The present report adds supporting evidence for the allosteric nature of the activation of fructose 1,6-bisphosphatase by monovalent cations by demonstrating its removal by chemical modification of arginyl residues under conditions that lead to no loss of fructose 1,6-bisphosphatase activity measured in the absence of monovalent cation activator. When modification was carried out in the presence of the allosteric inhibitor AMP, 8.2 arginyl residues were modified with a complete loss of the characteristic monovalent cation activation of fructose 1,6-bisphosphatase. These changes were completely prevented when modification was performed in the presence of AMP plus substrate, and the number of modified arginyl residues was reduced to 4.9. Thus, it may be concluded that the loss of monovalent cation activation of fructose 1,6-bisphosphatase was a consequence of the modification of three–four arginyl residues per mole of enzyme. Since the enzyme has four subunits, it is tempting to suggest that one arginyl residue per subunit is essential for the expression of monovalent cation activation of fructose 1,6-bisphosphatase. The term "essential" mentioned above is used here in the general terms defined by Means and Feeney (1971) as a group involved in, or in some way required for, a particular property. Therefore, the integrity of the modified arginyl residue could be either required for monovalent cation binding or for the conformational change induced by the monovalent cation. Although the former possibility cannot be completely excluded with the present data, the latter appears to be the most likely one. Therefore, it is suggested that the modified arginyl residue is essential for the enzyme conformational change induced by monovalent cations.

The protection exerted by the substrate fructose 1,6-bisphosphate against the loss of monovalent metal activation also merits some discussion, since it would appear improbable that this protection is exerted by direct blocking of the reactive arginyl residue essential for metal activation. Much more likely is the possibility that high concentrations of substrate (as used in the modification experiments) induce also a conformational change which renders unreactive the essential arginyl residue. Indeed, the SH titration experiment shown in Figure 4 clearly tends to support the latter explanation. In addition, results under several other experimental conditions (not detailed in the present report) appear to indicate that a close correlation exists between the reactivity of the highly reactive SH group and the reactivity (or exposure) of the arginyl residue essential for monovalent cation activation. Modification of fructose 1,6-bisphosphatase with butanedione under those conditions in which the fast reacting SH group is exposed (enzyme alone, or enzyme plus AMP, or enzyme plus $K^+$) led to the loss of monovalent cation activation, while under conditions which masked the fast reacting SH group (the same as above but in the presence of fructose 1,6-bisphosphate), no loss of monovalent cation activation was observed by modification of the enzyme with butanedione.

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