ALTERATION OF THE ALLOSTERIC PROPERTIES OF PHOSPHOFRUCTOKINASE BY MODIFICATION OF A SINGLE THIOL GROUP

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It has been established that P-fructokinase represents an important control point in glycolysis (Lowry, et al., 1964). The enzyme displays cooperativity with regard to one of its substrates, fructose-6-P (Uyeda and Racker, 1965) and thus may be classified as an allosteric enzyme (Monod, et al., 1965). In a previous paper (Kemp and Forest, 1968) we described the results of treating skeletal muscle P-fructokinase with dithiobis(2-nitrobenzoic acid) (DTNB). In those experiments the enzyme was assayed under optimal conditions and a decrease in activity was observed following reaction of the reagent with a single thiol group per enzyme protomer. As will be shown in this communication, when the enzyme is assayed at neutral pH and with low concentrations of fructose-6-P, the sulfhydryl modification produces a striking increase in enzyme activity. Evidence is presented that indicates that the chemical modification of a single thiol group results in an almost complete loss of the kinetic expression of cooperative interactions.

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METHODS

P-fructokinase was prepared from rabbit skeletal muscle as described by Kemp and Forest (1968) and recrystallized twice. Immediately before using the enzyme, $(NH_4)_2SO_4$ and bound nucleotide were removed and the protein concentration determined as described previously (Kemp and Krebs, 1967). The enzyme was treated with DTNB at 25° and pH 7.0 by mixing equal volumes of a solution of P-fructokinase (2 mg/ml) and a solution containing the indicated amount of DTNB. The weight of the protomer of P-fructokinase is assumed to be 90,000 g/mole (Kemp and Krebs, 1967; Paetkau and Lardy, 1968; Kemp and Forest, 1968), and calculations of the amount of DTNB to be used were based on this protomer weight. The enzyme was incubated with DTNB for 10 min at 25°. For assay, the enzyme was diluted to the appropriate concentration in a buffer containing 25 mM β-glycero-P, 25 mM glycylglycine, 0.01% bovine serum albumin, 1 mM EDTA at pH 7.0. Assays were performed by coupling the P-fructokinase reaction to the oxidation of DPNH with the use of fructose-1,6-diP aldolase, triosephosphate isomerase, and α-glycero-P dehydrogenase. The reaction mixture at pH 7.0 and 25° consisted of 25 mM β-glycero-P, 25 mM glycylglycine, 0.01% bovine serum albumin, 1 mM EDTA, 6 mM $MgCl_2$, 2 mM ATP, auxilliary enzymes, DPNH, and fructose-6-P as indicated.

RESULTS

Figures 1 A and B indicate the effect of increasing concentrations of DTNB on the activity of P-fructokinase. The reaction of DTNB with the enzyme goes to completion under the conditions described (Kemp and Forest, 1968). It can be seen that the activity of the enzyme when assayed at high substrate concentrations decreases rather regularly upon further additions with DTNB. On the other hand, the activity of the enzyme at low concentrations of fructose-6-P increases and reaches a maximum when 1 mole of DTNB per mole of protomer has reacted with the enzyme. Because the protomer of P-fructokinase has a single thiol group that is at least 50 times as reactive toward DTNB

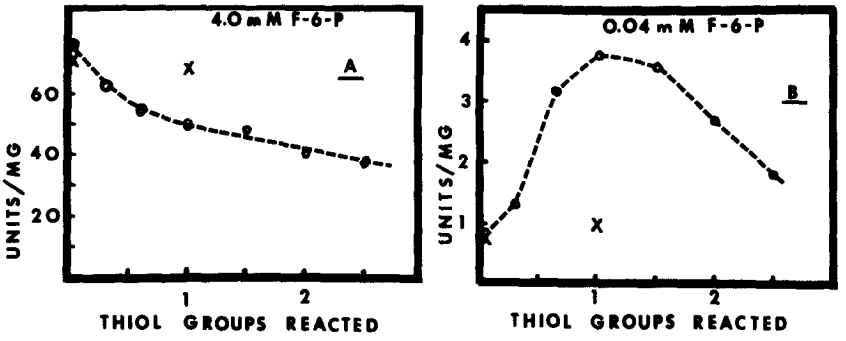

Figure 1A: Activity of P-fructokinase at 4.0 mM fructose-6-P following reaction with varying amounts of DTNB. Other conditions are described in the text. Experimental values indicated by X were obtained by assaying the modified enzyme after exposure to 0.1 mM dithiothrietol.

Figure 1B: Activity of P-fructokinase at 0.04 mM fructose-6-P following reaction with varying amounts of DTNB. Conditions are described in the text. Experimental values indicated by X were obtained by assaying the modified enzyme after exposure to 0.1 mM dithiothrietol. Variations of up to ± 15% of the values indicated were observed. The values shown are averages of from 2 to 5 determinations.

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than any other thiol group of the protein, it can be assumed that when 1 mole of reagent is present the reaction with this highly reactive thiol group is quantitative (Kemp and Forest, 1968). Thus, when one sulfhydryl group of P-fructokinase is modified by a disulfide interchange reaction with DTNB, the activity of the enzyme at 40 μM fructose-6-P increases more than 5-fold. Furthermore, the data in Figure 1 also show the modification of the enzyme can be easily reversed by the addition of dithiothreitol.

It is of interest to examine the saturation curve for fructose-6-P with the native and modified enzyme. Figure 2A (solid curve) describes the familiar sigmoid response of the enzyme to increasing concentrations of

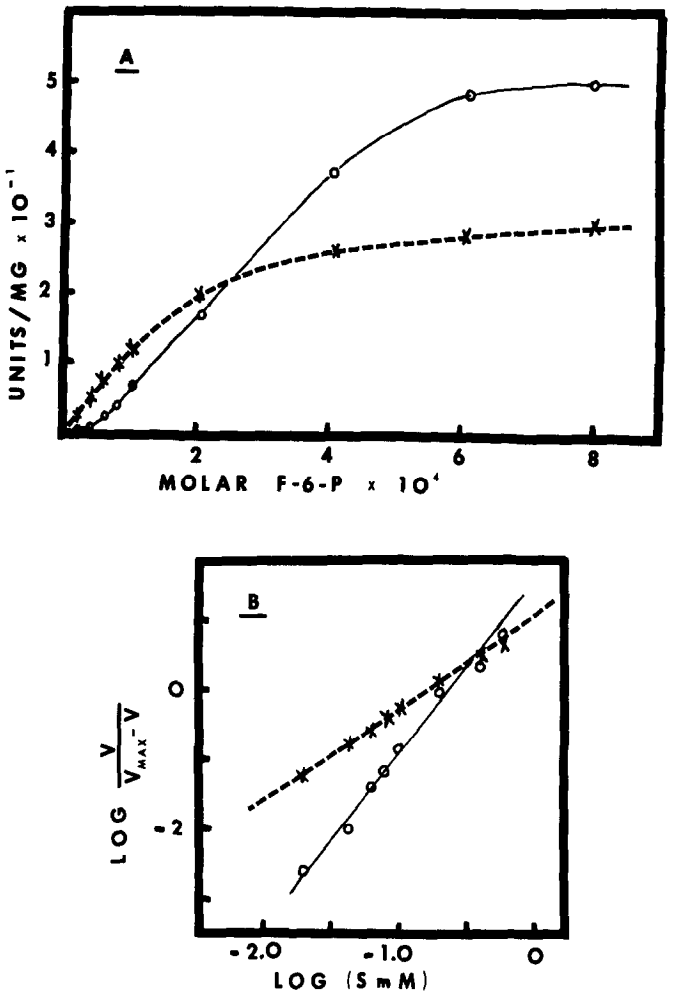

Figure 2A: Initial velocity vs. fructose-6-P for native P-fructokinase (solid curve) and P-fructokinase with one sulfhydryl group modified (dashed curve). Assay conditions are described in text.

Figure 2B: Hill plot corresponding to Fig. 2A.

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fructose-6-P that is typical of enzymes that display cooperative interactions. When the single most reactive thiol group of P-fructokinase is reacted with DTNB, the behavior of the enzyme is altered as described by the dashed curve. The maximum velocity is reduced as the result of the thiol modification, but what is most striking is the almost complete abolition of apparent cooper-

ative interactions. A Hill plot of the data (Figure 2B) indicates a decrease in the Hill constant from 2.5 to 1.3.

DISCUSSION

The foregoing results show that a single thiol group in P-fructokinase is essential with regard to the cooperative interactions displayed by the enzyme. At present it is not possible to describe how the chemical modification brings about the observed changes. It has been previously shown that the enzyme will bind 3 moles of ATP for every mole of fructose-6-P that can be bound by the protomer (Kemp and Krebs, 1967). One might propose that the binding of ATP at an inhibitor site on the enzyme is partially or completely blocked in the modified enzyme. This idea is supported by a recent observation that the thiol group in question is protected from reaction with DTNB in the presence of $[ATP\text{-}Mg]^{2-}$ but not by ATP^{4-} or Mg^{2+} alone (R.G. Kemp, unpublished results). It is hoped that further kinetic and equilibrium binding studies of the modified P-fructokinase will shed more light on the nature of the complex interactions displayed by this enzyme.

The observation that the reversible modification of this unique thiol group leads to a striking increase in activity at concentrations of fructose-6-P found *in vivo* (Lowry, et al., 1964) suggests a possible way of modulating the activity of the enzyme within the cell. If a naturally occurring disulfide present in the cell would react with P-fructokinase, an increase in the rate of flux through this key regulatory enzyme would be observed. The first such substance that such speculation brings to mind is insulin, particularly since it appears that insulin increases the activity of P-fructokinase in frog muscle (Özand and Narahara, 1964). We have been unable, however, to demonstrate any effect of this hormone on P-fructokinase when tested under a variety of conditions.

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