# Isotope-exchange evidence for allosteric regulation of hexokinase II by glucose 6-phosphate and for an obligatory addition of substrates

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The mechanism of rat skeletal-muscle hexokinase II has been shown by several initial-rate studies carried out by Fromm and his colleagues, using partially purified enzyme preparations, to proceed by a ternary-complex mechanism (e.g. Hanson & Fromm, 1967). Furthermore, these authors interpreted alternative-substrate studies as evidence for a rapid random addition of glucose and ATP, and these conclusions were supported by product-inhibition studies of ascites-tumour mitochondrial hexokinase II by Kosow & Rose (1968). However, this type of kinetic evidence could be given alternative interpretations, and does not provide unambiguous evidence for a rapid random

mechanism (e.g. Ricard *et al.*, 1972; Colowick, 1973). Although evidence for the formation of binary complexes between glucose and hexokinase II has been provided by the protection of the enzyme by glucose against proteolytic digestion, no similar effect could be demonstrated with ATP (Grossbard & Schimke, 1966).

To resolve the question of the mechanism of substrate addition, rat skeletal-muscle hexokinase II, purified to homogeneity by the method of Holroyde & Trayer (1976) was studied by the flux-ratio method introduced by Britton (1966). The ratio of the flux of [$^{14}$C]glucose 6-phosphate to glucose over the flux of glucose 6-[$^{32}$P]phosphate to ATP measured at constant [ADP] and [glucose 6-phosphate] concentrations in the absence of ATP as [glucose] was increased was found to be independent of [glucose] and equal to unity. This provided evidence for an obligatory order of addition of glucose and ATP, with glucose binding to the free enzyme. The dependence of the ratio flux of glucose 6-phosphate to ATP/flux of glucose 6-phosphate to glucose on [ATP] was consistent with an ordered mechanism in which ATP was the second substrate. Furthermore, the slope of the plot of this ratio against [ATP] increased with [ATP] and with [glucose 6-phosphate]. This finding was consistent with the binding of glucose 6-phosphate to an allosteric site on hexokinase II.

There is some controversy in the literature concerning the mode of feedback inhibition of the hexokinases by glucose 6-phosphate. Kinetic evidence obtained by Ning *et al.* (1969) and Purich & Fromm (1971) supports action of glucose 6-phosphate at the catalytic site and of ADP at a regulatory site, whereas Kosow & Rose (1970) argue that glucose 6-phosphate acts at an allosteric site and ADP at the catalytic site. Binding of glucose 6-phosphate to hexokinase I has been shown to cause a conformational change reflected in the protection of 8 out of 12 thiol groups that react with 5,5'-dithiobis-(2-nitrobenzoate) in the absence of glucose 6-phosphate and protects completely against inactivation by the reagent (Redkar & Kenkare, 1972). Glucose 6-phosphate has also been shown to release mitochondrially bound hexokinases in a specific manner, presumably through conformational changes. However, there is little evidence that an allosteric site is involved, even though this seems to be the most popular view. The unusual dependence of the ratio flux of glucose 6-phosphate to ATP/flux of glucose 6-phosphate to glucose on both [ATP] and [glucose 6-phosphate] provides kinetic evidence for such an allosteric site.

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