Difference in Kinetic Properties between Hexokinase Type I Isoenzymes from Various Rat Tissues with Reference to the Effect of a Thiol Inhibitor

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Hexokinase isoenzyme type I was purified from various rat tissues, and was subjected to kinetic studies in the presence or the absence of *p*-chloromercuribenzenesulphonate. The mode of the inhibition by the thiol inhibitor was different for the type I isoenzymes obtained from different tissues, suggesting that the type I isoenzymes from different tissues were not identical proteins.

Since the successful separation of hexokinase (ATP-D-hexose phosphotransferase, EC 2.7.1.1) isoenzymes in animal cells into four major types by starch-gel electrophoresis as well as by DEAE-cellulose column chromatography (Katzen & Schimke, 1965), the regulatory mechanism for each of the isoenzymes has been examined (McLean *et al.*, 1966; Katzen, 1967; Katzen *et al.*, 1970; Murakami & Ishibashi, 1972). Those studies seem to be based on the assumption that each of the isoenzyme types is identical throughout the body. Actually, for instance, the K_m value for glucose is almost the same for each type irrespective of the tissue from which the isoenzyme is obtained (Schimke & Grossbard, 1968). In the course of studies on the hexokinase isoenzymes in rat tissues, however, we found that partially purified type I preparations from various tissues differed markedly from each other in sensitivity to a thiol inhibitor, though they were almost identical in electrophoretic mobility on cellulose acetate and in their K_m values for glucose (Murakami *et al.*, 1973). This fact suggested that the type I isoenzymes from various tissues might not be completely identical proteins. To elucidate the mechanism of the difference, kinetic properties of the type I isoenzyme were comparatively examined with reference to the effect of a thiol inhibitor.

Experimental

The liver, skeletal muscle, kidney, spleen and brain were obtained from male rats (Donryu strain) which were killed by exsanguination from the carotid artery. Granuloma tissue, induced in the rats by the subcutaneous injection of carrageenin solution in the back, was also used as a source for the isoenzyme preparation (Murakami & Ishibashi, 1972). Each of the tissues was minced and homogenized with 1 vol. (or 2 vol. for the skeletal muscle) of 0.1 M-potassium phosphate buffer (pH 7.0) containing 10 mM-glucose and 5 mM-EDTA, chilled on ice, and the supernatant

fraction was obtained by centrifugation of the homogenate at 100000g (r_{av} 5.4 cm) for 60 min.

Hexokinase type I was purified from the supernatant fraction by precipitation with $(NH_4)_2SO_4$ and repeated column chromatography on DEAE-Sephadex A-50 and Sephadex G-200, by the procedure of Grossbard & Schimke (1966) with some modifications such as the use of DEAE-Sephadex instead of DEAE-cellulose. Hexokinase activity was measured by the method of Walker (1963). The hexokinase isoenzyme pattern was examined by electrophoresis and subsequent development on cellulose acetate membranes, by the method reported by Sato *et al.* (1969) with some modifications (Murakami & Ishibashi, 1972).

Results and discussion

In the present study, hexokinase type I isoenzymes in the supernatant fraction were examined, though some tissues, especially brain, contained the particulate form abundantly, since previous studies revealed no essential difference in the enzymological properties between soluble and particulate hexokinase (Tuttle & Wilson, 1970; Wilson, 1972). The isoenzymes were purified nearly 100-fold in specific activity by the above procedure, compared with the original homogenate solution. Most of the coexisting proteins and low-molecular-weight substances were removed from the partially purified isoenzyme preparations, but polyacrylamide-disc-gel electrophoresis revealed the presence of some contaminant proteins. The absence of hexokinase isoenzymes other than type I from these preparations was confirmed by cellulose acetate membrane electrophoresis.

Kinetic studies were made on these hexokinase type I isoenzyme preparations in either the presence or the absence of *p*-chloromercuribenzenesulphonate. Since the sensitivity to the thiol inhibitor differed between type I isoenzymes, as reported by Murakami *et al.* (1973), the concentration of

inhibitor was chosen to inhibit the activity to approx. 60–80% of the control in each case.

The K_m values for glucose of type I isoenzyme from liver, spleen and granuloma were $2.0 \times 10^{-5} \pm 0.3 \times 10^{-5}$ M, $3.2 \times 10^{-5} \pm 0.5 \times 10^{-5}$ M and $5.6 \times 10^{-5} \pm 0.7 \times 10^{-5}$ M respectively. These values coincided well with those previously reported for type I isoenzymes (Schimke & Grossbard, 1968). The addition of *p*-chloromercuribenzenesulphonate at $0.17 \mu\text{M}$ to liver type I isoenzyme, and at 84 nM to type I isoenzyme from the spleen and granuloma, caused a decrease in the velocity without appreciable change in the K_m value, as shown in Fig. 1(a–c). The double-reciprocal plots suggest that *p*-chloromercuribenzenesulphonate apparently acts as a non-competitive inhibitor on those hexokinase type I isoenzymes.

This was, however, not the case with type I isoenzyme from several other tissues. The K_m values for glucose of type I isoenzyme from skeletal muscle,

kidney and brain were $3.3 \times 10^{-5} \pm 0.3 \times 10^{-5}$ M, $5.0 \times 10^{-5} \pm 0.6 \times 10^{-5}$ M and $5.1 \times 10^{-5} \pm 0.8 \times 10^{-5}$ M respectively. The addition of *p*-chloromercuribenzenesulphonate at $0.25 \mu\text{M}$ to muscle and kidney type I isoenzyme, and at 33 nM to brain type I isoenzyme, caused an increase in the K_m value without significant change in the velocity, as shown in Fig. 1(d–f). The K_m values of type I isoenzyme from muscle, kidney and brain, in the presence of inhibitor at the above concentration, were calculated to change to $9.1 \times 10^{-5} \pm 1.3 \times 10^{-5}$ M, $1.3 \times 10^{-4} \pm 0.2 \times 10^{-4}$ M and $1.1 \times 10^{-4} \pm 0.1 \times 10^{-4}$ M respectively. The mode of the inhibitory effect of *p*-chloromercuribenzenesulphonate on the hexokinase type I isoenzymes from these three tissues seems to be competitive with glucose, thus differing from that on the isoenzymes from liver, spleen and granuloma.

Thus it could be deduced that hexokinase type I isoenzymes from various tissues of the rat were not

Fig. 1. Double-reciprocal plots for the velocity of hexokinase isoenzyme type I prepared from various rat tissues versus glucose concentration

For details see the text. ○, Control; ●, with *p*-chloromercuribenzenesulphonate (concn. in parentheses). (a) Liver ($0.17 \mu\text{M}$); (b) spleen (84 nM); (c) granuloma (84 nM); (d) skeletal muscle ($0.25 \mu\text{M}$); (e) kidney ($0.25 \mu\text{M}$); (f) brain (33 nM). v is expressed as $\Delta E_{340}/\text{min}$.

identical with each other, not only in the sensitivity to the thiol inhibitor as reported previously (Murakami *et al.*, 1973), but also in the mode of the inhibition. According to the mode, the isoenzymes could be classified into two groups, i.e. competitively and non-competitively inhibited. Though the underlying mechanism for the difference between the two groups has not yet been clarified, the positional relation between the inhibitor site and substrate site on the isoenzyme molecule might be different between the two groups. Thus the present results may be related to those reported by Redkar & Kenkare (1972) for brain mitochondrial hexokinase. They described that glucose was protective against the inactivating action of 5,5'-dithiobis-(2-nitrobenzoic acid), another thiol inhibitor. On the other hand, the existence of other hexokinase isoenzymes in the same tissue might be involved in the difference, since apparent competitive inhibition by *p*-chloromercuribenzenesulphonate was observed for type I isoenzymes obtained from the

tissues containing type III isoenzymes, whereas non-competitive inhibition was observed for type I isoenzymes from the tissues lacking type III isoenzyme, as far as the tissues examined were concerned.

The explanation that the difference might be due to the coexisting thiol compounds and proteins seems to be unlikely, since almost all of them had already been removed from the isoenzyme preparations examined during the purification procedure, as mentioned above.

Such a difference, however, was not observed in the similar kinetic studies on some of the hexokinase type I isoenzymes, with another substrate, ATP. The K_m values for ATP of type I isoenzyme from spleen, granuloma and brain were $9.1 \times 10^{-4} \pm 1.1 \times 10^{-4}$ M, $5.0 \times 10^{-4} \pm 0.4 \times 10^{-4}$ M and $5.5 \times 10^{-4} \pm 0.6 \times 10^{-4}$ M respectively. These values also coincided well with those reported previously (Schimke & Grossbard, 1968). The addition of *p*-chloromercuribenzenesulphonate at $0.83 \mu\text{M}$ to type I isoenzyme from spleen and granuloma, and at 67 nM to brain type I isoenzyme, caused an increase in the K_m value for ATP without significant change in the velocity, as shown in Fig. 2. The K_m values of type I isoenzyme from spleen, granuloma and brain were changed by the addition of the inhibitor to $2.5 \times 10^{-3} \pm 0.4 \times 10^{-3}$ M, $7.7 \times 10^{-4} \pm 0.6 \times 10^{-4}$ M and $8.3 \times 10^{-4} \pm 1.4 \times 10^{-4}$ M respectively. For ATP the mode of inhibition by *p*-chloromercuribenzenesulphonate was apparently all competitive as far as those type I isoenzymes were concerned, as shown in Fig. 2.

Such a finding may shed some light on the state of binding sites for glucose and ATP on the hexokinase isoenzyme molecule, as the reaction mechanism of the enzyme with the substrates is considered to be of random type (Bachelard *et al.*, 1971). The role of Mg^{2+} (Bachelard, 1971; Purich & Fromm, 1972) may also be explicable by the reactivity of the thiol groups.

The present results seem to differ from those reported by Parry & Walker (1967), who found for rat hepatic glucokinase (EC 2.7.1.2) that the inhibition by several thiol inhibitors was non-competitive with respect to ATP but competitive with respect to glucose. Such a difference may lead to the further elucidation of the relationship between hexokinase and glucokinase.

Fig. 2. Double-reciprocal plots for the velocity of hexokinase isoenzyme type I prepared from spleen (a), granuloma (b) and brain (c) versus ATP concentration

For details see the text. $\circ$, Control; $\bullet$, with *p*-chloromercuribenzenesulphonate: $0.83 \mu\text{M}$ in (a) and (b), 67 nM in (c). v is expressed as $\Delta E_{340}/\text{min}$.

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