Discrimination of Antigenic Site and Thiol-Inhibitor-Sensitive Site of Hexokinase Isoenzymes

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Rabbit antiserum was prepared against hexokinase isoenzyme type I which was purified from rat brain mitochondria. The antiserum inhibited the activity of the mitochondrial hexokinase type I as well as that of the cytosolic type I enzyme prepared from rat brain, kidney and spleen. It did not, however, inhibit the activity of type II hexokinase from muscle and spleen or that of the type III enzyme from spleen. The results suggest that all hexokinase type I isoenzymes may have a common antigenic site irrespective of their sources, though their responses to a thiol inhibitor are different.

Mammalian hexokinases (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1) can be separated into four major types of isoenzymes. The enzymological, electrophoretic and chromatographic properties of each isoenzyme type are characteristic and independent of tissue source (Schimke & Grossbard, 1968). We found, however, that the response to a thiol inhibitor, *p*-chloromercuribenzenesulphonate, was different for type I isoenzymes in the cytosol fractions of various rat tissues (Murakami *et al.*, 1973; Kamikashi *et al.*, 1974). On the other hand, it is known that hexokinase in the brain is predominantly mitochondrial, though the enzyme is generally located in the cytosol in other tissues. In the previous study, we found that the mitochondrial and cytosolic hexokinases in the rat brain differed in their response to the thiol inhibitor, diabetes and insulin administration, though both were judged to be type I from the above-mentioned criteria (Ouchi *et al.*, 1974). It is thus of some interest that the relationship, both molecular and functional, between isoenzymes originating from different tissues and subcellular fractions should be elucidated. We have attempted in the present study to investigate this problem by using antiserum directed against hexokinase type I from rat brain mitochondria.

Experimental

Donryu-strain rats were used for the preparation of the mitochondrial hexokinase. They were killed by exsanguination and the entire brains were removed and pooled in ice-chilled 20mM-Tris-HCl buffer (pH 7.4) containing 250mM-sucrose and 10mM-glucose. The brains were homogenized in the same buffer and the mitochondrial fraction was obtained by the conventional differential-centrifugation method (Katzen *et al.*, 1970).

Purity of the mitochondrial fraction was checked by measuring the activity of three marker enzymes,

glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) (Langdon, 1966), succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1] (Bonner, 1955) and NADH-cytochrome *c* reductase [NADH-(acceptor) oxidoreductase, EC 1.6.99.3] (Mahler, 1955).

Mitochondrial hexokinase was solubilized from the mitochondria by incubating at 30°C for 20 min in the above-mentioned buffer with glucose 6-phosphate added to a final concentration of 1mM. Further purification of the mitochondrial hexokinase was carried out by the procedure of Chou & Wilson (1972), with some modification (DEAE-Sephadex A-50 instead of DEAE-cellulose).

Cytosolic hexokinase isoenzymes were also purified from the brain, kidney, spleen and skeletal muscle of rats by the method reported previously (Grossbard & Schimke, 1966; Murakami & Ishibashi, 1972).

Hexokinase activity was measured by the method of Walker (1963); the hexokinase isoenzyme pattern was examined as reported in previous papers (Sato *et al.*, 1969; Murakami & Ishibashi, 1972), and protein was determined fluorimetrically (Hiraoka & Glick, 1963).

Purified rat brain mitochondrial hexokinase was concentrated and dialysed against 0.9% NaCl. This enzyme solution (4ml), containing approx. 0.3mg of protein (Hiraoka & Glick, 1963), was homogenized with an equal volume of complete Freund's adjuvant, and the emulsion was injected intradermally into an albino rabbit as recommended by Vaitukaitis *et al.* (1971). Then 3 weeks later, the rabbit was injected again with about 1mg of the hexokinase freshly prepared in the same way. The rabbit was kept for another 3 weeks, and blood was drawn from the marginal ear vein. The antiserum thus obtained was divided into equal small portions and stored at -20°C.

Results and discussion

The mitochondrial fraction prepared had a satisfactorily high activity of the mitochondrial marker enzymes and negligible activity of the cytosolic marker enzyme. From the activity it was calculated that contamination by the cytosol in this fraction was less than 5%. By incubating this mitochondrial fraction in the presence of glucose 6-phosphate (Rose & Warms, 1967; Chou & Wilson, 1972), 70–80% of the activity of the mitochondria-bound hexokinase was released. The mitochondrial hexokinase thus obtained was judged as the isoenzyme type I from the electrophoretic mobility and K_m values for glucose and ATP, all of which are quite similar to those of the cytosolic counterpart (Schimke & Grossbard, 1968; Ouchi *et al.*, 1974). The specific activity of the final preparation of the purified mitochondrial hexokinase after the DEAE-Sephadex chromatography was 52 units ($\mu\text{mol}/\text{min}$)/mg of protein, this value being assumed to be sufficiently high compared with that of the purified glucokinase (Grossman *et al.*, 1974) if molecular weights are taken into consideration. In accordance with such a specific activity, polyacrylamide-gel electrophoresis revealed a single protein band, the location of which coincided with that of the hexokinase activity. This mitochondrial hexokinase preparation was used for the immunization of a rabbit.

The presence of the anti-hexokinase antibody in the serum of the immunized rabbit was proved by the formation of a single precipitin band on agar by the Ouchterlony double-diffusion method and by the precipitation reaction.

The activity of the mitochondrial hexokinase type I from the brain was decreased by the addition of 100 μl

of the antiserum to about 38% of that with added control serum. The activity of the cytosolic type I isoenzymes prepared from the brain, kidney and spleen of rats was also decreased to about 42, 41 and 42% respectively. In other words, the extent of the inhibition was almost the same for all the type I isoenzymes examined. It should be pointed out that these type I isoenzymes were found in the previous studies to be different in their response to *p*-chloromercuribenzenesulphonate, both with respect to sensitivity and mode of inhibition (Murakami *et al.*, 1973; Kamikashi *et al.*, 1974; Ouchi *et al.*, 1974). On the other hand, the activity of the hexokinase type II isoenzymes from skeletal muscle and spleen and that of the type III isoenzymes from spleen were not inhibited by the antiserum at all. In accordance with the effect on the activity, a precipitin band appeared between the antiserum and the hexokinase type I preparations irrespective of their origin, whereas such a band was not formed between the antiserum and the type II and III isoenzymes. These results coincide with those reported on human hexokinases (Heumann *et al.*, 1974).

As to the mode of the inhibition by the antiserum, both mitochondrial and cytosolic hexokinase type I isoenzymes from the rat brain were inhibited non-competitively with respect to ATP as the substrate (Fig. 1), though it was demonstrated in the previous studies that the former isoenzyme was inhibited non-competitively whereas the latter was inhibited competitively by the thiol inhibitor (Kamikashi *et al.*, 1974; Ouchi *et al.*, 1974). Further, the mode of inhibition by the thiol inhibitor of the two brain type I isoenzymes which had been partially inhibited by the preincubation with the antiserum was the same as observed in the previous study (Kamikashi *et al.*,

Fig. 1. Double-reciprocal plots of the velocity of hexokinase isoenzyme type I prepared from rat brain cytosol (a) and mitochondria (b) versus ATP concentration

For details see the text. ●, Control; ○, with the antiserum. v is expressed as $\Delta E_{340}/\text{min}$.

Fig. 2. Double-reciprocal plots of the velocity of hexokinase isoenzyme type I prepared from rat brain cytosol (a) and mitochondria (b) versus ATP concentration, after pretreatment with the antiserum

For details see the text. $\square$, Control (antiserum alone); $\blacksquare$, with *p*-chloromercuribenzenesulphonate ($2.5\mu\text{M}$ for the cytosol type I and $10\mu\text{M}$ for the mitochondrial type I). v is expressed as $\Delta E_{340}/\text{min}$.

1974; Ouchi *et al.*, 1974) (Fig. 2). These findings seem to suggest a distinction between the site attacked by the antiserum and that attacked by the thiol inhibitor.

Our results therefore indicate that hexokinase isoenzyme type I can be distinguished immunologically from the type II and III isoenzymes with the use of antiserum against purified mitochondrial hexokinase from rat brain, though all of these three types were reported to have similar molecular weight (Schimke & Grossbard, 1968; Purich *et al.*, 1973). On the other hand, all of the type I isoenzymes were found to have the same antigenic site irrespective of the originating tissues and subcellular fractions, in spite of the dissimilarity of their response to the thiol inhibitor. The nature of the inhibition of hexokinase by the thiol inhibitor, especially of the competitive inhibition, has been discussed (Parry & Walker, 1967; Kamikashi *et al.*, 1974) but has not been fully elucidated so far. If the competitive relationship between the substrate and inhibitor is, as discussed by Parry & Walker (1967), due to the effect of the substrate on the rate at which an enzyme thiol group undergoes an essentially irreversible reaction to form a covalently bonded complex with the inhibitor, such an inhibition may indicate the presence of, at least, a thiol group around the substrate-binding site. For yeast hexokinase, the presence of a cysteine residue in the glucose-binding site was reported (Bessel, 1973). Then it may be deduced that the antiserum attacks the site that is common between the type I isoenzymes of the rat tissues and is different from the substrate-binding site, at least in cases where the hexokinase activity is inhibited competitively by the thiol inhibitor.

It was difficult to examine the mode of the inhibition with respect to glucose, because of the presence of glucose in the antiserum.

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