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Active-Site Thiol Groups of Yeast Hexokinase

By J. G. JONES. (*Department of Biochemistry, University College, Cathays Park, Cardiff*)

Two forms of hexokinase (A and B) can be isolated from baker's yeast by a method that avoids proteolytic degradation of the enzymes (Lazarus, Ramel, Rustum & Barnard, 1966; Gazith, Schulze, Gooding, Womack & Colowick, 1968). Inactivation of hexokinase B with iodoacetate at low ionic strength was accompanied by the alkylation of all 8 thiol groups/molecule of mol.wt. 100000 (Lazarus, Derechin & Barnard, 1968). The present paper describes the alkylation of hexokinase B at high ionic strength.

Hexokinase B was reacted with iodoacetate (1–10mM) at 35° and pH 8.6 in glycine–NaCl–NaOH buffer, 1.0M, when the enzyme is known to exist as a dimer of mol.wt. approx. 50000 (Derechin, Ramel, Lazarus & Barnard, 1966). Inactivation of the enzyme is first-order with respect to the concentration of each reactant with a rate constant of $0.131 \text{ mole}^{-1} \text{ sec}^{-1}$. The rate of inactivation increases abnormally sharply at pH values above 8.5, probably owing to a progressive unfolding of the protein with increasing pH.

The enzyme is protected from this inactivation by the presence of glucose and, from these data, the dissociation constant for the enzyme–glucose complex has been calculated to be 0.46mM. This value compares favourably with that determined by equilibrium dialysis (Gazith *et al.* 1968).

Alkylation of hexokinase B, in the same glycine buffer, by iodo[^{14}C]acetate shows that inactivation follows closely the incorporation of 2 atoms of ^{14}C /molecule of enzyme. A further 2 atoms of ^{14}C /molecule are incorporated at a lower rate (k $0.011 \text{ mole}^{-1} \text{ sec}^{-1}$).

Analysis of the protein, after incorporation of 4 atoms of ^{14}C /molecule, identified over 90% of the radioactivity as *S*-carboxymethylcysteine; no free cysteine could be detected in hydrolysates of the alkylated protein.

The collective results indicate that 2 thiol groups/molecule (mol.wt. 50000) of hexokinase B are probably located at the active site. These essential thiol groups may be preferentially labelled with iodo[^{14}C]acetate and this technique may be a useful basis for investigating amino acid sequences in the active site of the enzyme.

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Enzyme Distributions in an Amoeba During Encystment

By S. M. BOWEN, A. J. GRIFFITHS and D. LLOYD. (*Department of Microbiology, University College of South Wales and Monmouthshire, Cardiff CF1 3NR*)

The subcellular distributions of enzymes were studied in vegetative amoebae of *Hartmannella castellanii* and also during encystment in a replacement medium (Griffiths & Hughes, 1968). The cells were broken in an all-glass hand-operated homogenizer in 0.32M-sucrose–20mM-tris–10mM-EDTA medium. The cell-free homogenate was centrifuged at 35000 rev./min. for 2½ hr. ($\approx 100000g$ for 1 hr.) in an unbuffered sucrose gradient [15–60% (w/v) sucrose] in a BXIV zonal rotor.

In vegetative amoebae 44% of the acid phosphatase was sedimentable with a median buoyant density of 1.16g./ml., as was 76% of the catalase (median buoyant density 1.21) and all the recoverable cytochrome oxidase activity (median buoyant density 1.18). Of the other enzymes studied only 28% of the *N*-acetyl- β -glucosaminidase (1.15) and 27% of the NADPH–cytochrome *c* oxidoreductase (1.19) but 67% of the NADH–cytochrome *c* oxidoreductase were sedimentable.

After incubating the amoebae in the encystment medium acid phosphatase and *N*-acetyl- β -glucosaminidase showed increases in both their sedimentability (69% and 40% respectively) whereas the sedimentability and median buoyant density of catalase increased only after an initial decrease during the first 5 hr. of encystment. After 15 hr. encystment the sedimentable acid phosphatase and *N*-acetyl- β -glucosaminidase were both resolvable as double peaks (median buoyant densities 1.16 and 1.19 for acid phosphatase and 1.16 and 1.20 for 1.19 for acid phosphatase and 1.16 and 1.20 for *N*-acetyl- β -glucosaminidase).

An increase also occurred in the median buoyant density of cytochrome oxidase (1.20) after 15 hr. encystment, but its sedimentability remained

constant at 100%. No changes were observed in the median buoyant densities of either NADH- or NADPH-cytochrome *c* oxidoreductase, but the sedimentable fractions of both were decreased (22% and 16% respectively).

The specific activities of these enzymes also showed interesting changes during encystment. Increases were observed in the activities of *N*-acetyl- β -glucosaminidase, catalase and NADH- and NADPH-cytochrome *c* oxidoreductases, whereas acid phosphatase activity decreased throughout encystment. There were no changes in cytochrome oxidase activity.

These results will be discussed in their relation to the physiological and structural changes that are known to occur in amoebae during cellular differentiation.

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An Assay for Liver Glutamine Aminotransferase

By P. J. BUTTERRY*, E. V. ROWSELL and J. A. CARNIE. (*Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester 1*)

In glutamine aminotransferase reactions 2-oxoglutarate formed is rapidly hydrolysed to 2-oxoglutarate and ammonia (Meister, 1965), and commonly glutamine aminotransferase has been assayed in crude tissue preparations by measuring the increase in ammonia formation from glutamine after keto acid addition, i.e. correcting for glutaminase activity (e.g. Beaton, 1954).

There are disadvantages to this procedure: difficulties may be encountered in measuring small amounts of ammonia; the increase in ammonia production with keto acid may be only a small proportion of that for glutamine alone; and spurious aminotransferase activity will be recorded if glutaminase is activated by the keto acid (see, e.g., Kvamme, Tveit & Svenneby, 1965).

We have assayed by measuring the accumulation of 2-oxoglutarate. Incubations at 37° under nitrogen included 0.02M-L-glutamine, 0.02M-2-oxoisocaproate (4-methyl-2-oxopentanoate), 0.05M-sodium borate buffer, pH 7.8, and liver homogenate. Heat-inactivated samples were analysed spectrophotometrically for 2-oxoglutarate with glutamate dehydrogenase. With ammonia and glutamate dehydrogenase, 2-oxoisocaproate itself slowly re-oxidizes NADH; nevertheless on assaying 2-oxoglutarate standards in the presence of equal

samples of a heat-inactivated aminotransferase incubation linear calibration curves were obtained with the expected increments, measuring extinction decreases (at 340nm.) for the 5 min. period after the addition of glutamate dehydrogenase.

Liver contains glutaminase, and liberated glutamate could produce 2-oxoglutarate by transamination with keto acids, giving an overestimation of glutamine aminotransferase activity. Borate buffer was employed to minimize glutaminase activity (Roberts, 1960), and 2-oxoisocaproate since it is a poor substrate for liver transamination with glutamate but among the most active with glutamine (Meister & Tice, 1950).

But it was essential to obtain some measure of the glutamate contribution. A glutamine aminotransferase incubation was made with rat liver, and a similar incubation without glutamine but with glutamate injected at a rate simulating glutaminase activity (based on rat liver assays). Rates of 2-oxoglutarate formation were 19 and 1.5 μ moles/hr./g. of liver from glutamine and glutamate respectively. With hamster liver comparable rates were 27.5 and 1.8 μ moles/hr./g.

Negligible 2-oxoglutarate production was observed with glutamine or 2-oxoisocaproate as sole substrate.

Formation of leucine (ninhydrin estimation after separation on paper) and 2-oxoglutarate were equivalent in mouse liver assays; to the same extent ammonia formation (glutamate dehydrogenase estimation) exceeded that from glutamine alone.

Linear progress curves were obtained with incubations up to 90 min., giving rates proportional to liver concentration up to 1.5% (w/v) with mouse, rat or rabbit.

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* Present address: Department of Applied Biochemistry and Nutrition, University of Nottingham.