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Negative Co-operativity in Glutamate Dehydrogenase: Coenzyme-Binding Studies

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The active oligomer of ox liver glutamate dehydrogenase consists of six apparently identical polypeptide chains of molecular weight $5.7 \times 10^4 \pm 0.3 \times 10^4$ (Cassman & Schachman, 1971; Smith *et al.*, 1970). Glutamate enhances the fluorescence of enzyme-bound NADH and NADPH and also has a pronounced hypochromic effect on the absorption spectrum; spectrophotometric titrations as the abortive complex indicated one binding site per subunit for both reduced coenzymes and an active-centre equivalent weight of 5.7×10^4 (Egan & Dalziel, 1971). Kinetic studies of the oxidative deamination of glutamate showed a complex pattern of coenzyme activation with both NAD^+ and NADP^+ , suggesting negative interactions between the six active centres in the oligomer (Dalziel & Engel, 1968; Engel & Dalziel, 1969). Equilibrium dialysis studies of NAD^+ and NADP^+ binding in the presence of the substrate analogue glutarate established that coenzyme binding in the ternary complex could not be described in terms of six identical and independent binding sites (Dalziel & Egan, 1972). Either there are negative interactions between the subunits or there are at least two sets of subunits with different intrinsic dissociation constants for coenzyme. In the presence of the allosteric effector ADP the coenzyme binding was weakened and could be described by a single dissociation constant.

Further information to elucidate the complex overall kinetics and the possible role of negative co-operativity has been sought by 'burst' experiments by the stopped-flow method and by reduced-coenzyme-binding studies by fluorescence titrations, utilizing both titrations of enzyme with coenzyme and titrations of coenzyme with enzyme. Taken together, both kinds of titration show conclusively that in the binding of NADH either there are negative interactions between the six subunits or about half the active centres bind coenzyme with a dissociation constant of about $2 \mu\text{M}$ and the remainder with a dissociation constant of $20 \mu\text{M}$. In the presence of glutamate qualitatively similar results were obtained, indicating one coenzyme-binding site per subunit, but

coenzyme binding was greatly strengthened and could be described by two dissociation constants of about 0.03 and $0.4 \mu\text{M}$. In presence of the allosteric effector GTP the coenzyme-binding curve was sigmoid, and the presence of more than one binding site per subunit was indicated. The latter result supports conclusions from circular-dichroism measurements (Jallon & Iwatsubo, 1971; Koberstein & Sund, 1971) and shows that the fluorescence of NADH bound at the additional site is also enhanced. Since GTP and NADH together cause dissociation of aggregates of the oligomer, a possible explanation of the apparent positive interactions in coenzyme binding is that the free oligomer has greater affinity for NADH than has the molecular aggregate.

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Some Properties of Yeast Isocitrate Dehydrogenase

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The activity of yeast isocitrate dehydrogenase is regulated by AMP, which increases the affinity of the enzyme for its substrates without altering the maximal velocity. The co-operativity of NAD^+ binding is diminished by raised concentrations of AMP or isocitrate. However, AMP activation causes only a slight fall in the Hill coefficient for isocitrate despite a 12-fold increase in isocitrate-binding affinity (Atkinson *et al.*, 1965).

The enzyme can be purified to homogeneity with 50% overall recovery by chromatography on DEAE-cellulose followed by selective elution from CM-cellulose by AMP. The isoelectric point of the enzyme rises from 5.6 to 7.0 during purification. This change can be revealed by isoelectric focusing, and is responsible for marked alterations in the behaviour of the enzyme on ion-exchange celluloses. Isoelectric focusing also generates a number of 'isoenzymes' as artifacts. These sub-forms cannot be discerned in any separation system that avoids the use of carrier

ampholytes. The separated 'isoenzymes' show no differences in kinetic properties, and have identical electrophoretic mobilities. Attempts to re-focus the sub-forms give rise to additional bands.

The molecular weight of the enzyme estimated by gel filtration (375000) does not alter during purification. Ultracentrifugation experiments (Barnes *et al.*, 1971) indicate a molecular weight nearer 300000 for enzyme purified by another method.

The pure enzyme contains two types of subunit in equal numbers. Both types have a molecular weight of 40000, estimated by gel electrophoresis in sodium dodecyl sulphate, so the enzyme is probably an octamer. The subunits can be separated by electrophoresis on polyacrylamide gels in 8M-urea, and differ in amino acid composition and tryptic-peptide 'fingerprints'. Cysteine residues are found only in one type of subunit.

When excited at 290 nm the enzyme fluoresces at 340 nm. After the binding of NADH the emission

maximum shifts to 440 nm, and the 340 nm peak is suppressed. This allows a direct estimate of the NAD^{+} - and NADH-binding constants. Isocitrate binds to the enzyme-NADH complex and modifies the emission spectrum. Unfortunately the low binding affinities prevent an estimate of the number of active sites.

Results obtained from fluorescence titrations can be combined with new kinetic measurements to build up a model for the reaction mechanism. The minimal hypothesis needed to explain the experimental findings is an extended form of the allosteric theory of Monod *et al.* (1965) in which there are three rather than two interconvertible enzyme conformations.

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COMMUNICATIONS

Regulation of Granulose Synthesis in *Clostridium pasteurianum*

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Species of butyric clostridia synthesize granulose, an intracellular glucan whose structure resembles that of amylopectin (Gavard & Milhaud, 1952). In one strain of *Clostridium pasteurianum* maximal synthesis of granulose coincided with sporulation (Mackey & Morris, 1971), so that knowledge of the means of regulation of granulose biosynthesis could contribute to our understanding of the condition of the organism when sporulation is initiated.

Like glycogen synthesis in other bacteria (Preiss, 1969), granulose was synthesized in *Cl. pasteurianum* by the sequential action of an ADP-glucose pyrophosphorylase (EC 2.7.7.-) and an ADP-glucose-granulose transglycosylase (i.e. granulose synthetase). Throughout its exponential anaerobic growth in a glucose - ammonium - vitamins - salts medium (Mackey & Morris, 1971) wild-type *Cl. pasteurianum* N.C.I.B. 9486 synthesized considerable quantities of granulose (15% of the dry weight of the organism); under these conditions both enzymes were present at a specific activity of approx. 6nmol/min per mg of protein. Several granulose-negative mutant strains were isolated that grew in minimal medium at a simi-

lar rate to the wild-type. These lacked either ADP-glucose pyrophosphorylase or granulose synthetase.

The soluble ADP-glucose pyrophosphorylase was partially purified (33-fold) and was found to be specific in its utilization of ATP and glucose 1-phosphate. Its ATP-saturation curve was not sigmoidal (K_m 2mM for ATP) and its activity was not enhanced by phosphorylated glycolytic intermediates, pyruvate, NADPH or pyridoxal 5'-phosphate. It was inhibited only by relatively high concentrations of ADP (K_i 20mM); both AMP and inorganic orthophosphate were without effect.

Partial purification (22-fold) of the granulose synthetase was facilitated by its being bound to the particles of granulose isolated by low-speed centrifugation of crude extracts of *Cl. pasteurianum*. The enzyme proved to be specific for ADP-glucose and its activity was not enhanced by hexose phosphates; ADP and to a smaller extent AMP were inhibitory. Pyridoxal 5'-phosphate (1mM) was also inhibitory, suggesting the presence of an essential lysine residue at the catalytic site (Schnackerz & Noltmann, 1971).

These findings suggest that, although the rate of granulose biosynthesis will probably reflect the adenylate-energy-charge status of the organism (Shen & Atkinson, 1970), it is not subject to the fine control effected by allosteric activation of ADP-glucose pyrophosphorylase that is a feature of glycogen production in many bacteria (Preiss, 1969).

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