The mechanism, role and control of the inactivation of glutamate dehydrogenases in yeast

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Glutamate dehydrogenase (GDH) occupies a central role in the metabolism of many microorganisms because it provides a link between carbohydrate and nitrogen metabolism (Smith et al., 1975; Brown et al., 1974). Many simple eukaryotic micro-organisms possess two genetically distinct forms of GDH with different nucleotide specificities, a catabolic role being assigned to the NAD-specific enzyme, whereas the NADP-specific enzyme performs an anabolic function (Smith et al., 1975; Brown et al., 1974). Originally it was thought that the concurrent regulation of glutamate biosynthesis and degradation was achieved by rigorous control of the synthesis of NAD-GDH and NADP-GDH under different growth conditions (Stachow & Sanwal, 1967). It is now apparent that additional control mechanisms operate to regulate the activity of NAD-GDH and NADP-GDH (Hemmings, 1978a, b). This article discusses the role of protein phosphorylation in the regulation of NAD-GDH, and the role of specific enzyme degradation in regulating the activity of NADP-GDH.

Inactivation of NAD-dependent glutamate dehydrogenase

Growth of yeast (Candida utilis or Saccharomyces cerevisiae) on glutamate or some other amino acid leads to the de-repression (or induction?) of NAD-GDH (Hierholzer & Holzer, 1963; Ferguson & Sims, 1974). Starvation or the addition of either ammonia or glutamine to yeast cultures leads to a rapid loss of enzyme activity (Ferguson & Sims, 1974). The loss of enzyme activity is completely reversible even in the presence of protein-synthesis inhibitors (Hemmings & Sims, 1977).

The NAD-GDH was purified to homogeneity from yeast cultures taken before and after enzyme inactivation (Hemmings, 1978a, 1980). The purification involved chromatography on DEAE-cellulose, Cibacron Blue–agarose and gel filtration on Ultrogel AcA22. The properties of the two enzyme preparations were compared in an attempt to identify the mechanism of inactivation (Hemmings, 1978a, 1980). Both enzyme forms, i.e. the fully active and less active, of NAD-GDH had a molecular weight of 55000, a s20,w of 14 S, identical amino acid compositions, and are composed of four apparently identical subunits, of molecular weight 116000.

The catalytic properties of the two enzyme forms, however, were quite dissimilar. The less-active form of NAD-GDH isolated from glutamate-starved cultures contained about 1.25 mol of alkali-labile phosphate/mol of subunit. This phosphate group was apparently introduced into the enzyme during growth of yeast (Candida utilis or Saccharomyces cerevisiae) on glutamate or some other amino acid. These studies were extended with the availability of pure phospho- and dephospho-GDH. Both enzyme forms were cleaved by trypsin and chymotrypsin to yield a 64500 mol. wt. fragment and a 48000 mol. wt. fragment. Under conditions where only limited proteolysis occurs, the phospho-GDH could be completely re-activated; the activity of the dephospho-GDH was unaffected under similar conditions. Analysis of the products of limited proteolysis by using 32P-labelled GDH revealed that the phosphate group was located exclusively on the 48000 mol. wt. domain. Similar studies using limited proteolysis to probe the structure of NAD-GDH from Neurospora crassa have been carried out by Haberland et al. (1980). They also found that GDH could be cleaved into two domains, of mol. wts. 63000 and 47000, corresponding to an N-terminal and C-terminal portion of the enzyme respectively. Interestingly, the 63000-mol. wt. fragment contained considerable sequence homology to bovine NADP-GDH (mol. wt. of the subunit of this enzyme is 55000) and is thought to represent the catalytic domain. The 47000-mol. wt. fragment thus represents an N-terminal extension of the molecule and also appears to contain the phosphorylation site. Obviously the phosphorylation site must interact with the catalytic site on the 63000-mol. wt. fragment to cause a loss of enzyme activity.

The above experiments demonstrate that the rapid changes in activity of NAD-GDH observed during glutamate starvation are achieved by reversible phosphorylation. In order to gain a greater understanding of the regulation of NAD-GDH it was necessary to isolate and characterize the protein kinase(s) and protein phosphatase(s) that interconvert this enzyme between its active and less active states.

During the initial stage of the isolation of the phospho-GDH, it was noted that the enzyme in cell-free extracts was rapidly re-activated unless fractionated by DEAE-cellulose (Hemmings, 1981). Examination of the properties of the purified ‘activating factor’ revealed it to be a protein phosphatase (Hemmings, 1981). It is able to dephosphorylate, in a Mg2+-dependent manner, phospho-GDH, histone, casein and the synthetic peptide Kempbide, but is unable to hydrolyse non-protein phosphate esters such as p-nitrophenyl phosphate or ATP.

The phosphatase dephosphorylated and re-activated GDH that had been phosphorylated in vitro and in vivo (see below). Re-activation of phospho-GDH by the purified phosphatase showed similar properties to those observed in vivo; the pH optimum of the GDH shifted from 6.8 to 8.1 on incubation with the phosphatase. Most interestingly, the phosphatase appears to be regulated by substrate-directed metabolites. The rate of re-activation is stimulated 2–3-fold by the addition of glutamate and NAD+; no stimulation is observed when these two compounds are added singly. The substrates for the reductive amination reaction of GDH, 2-oxoglutarate, NH3 and NAD+, do not affect the rate of dephosphorylation.

The isolation of the protein kinase that could phosphorylate and inactivate NAD-GDH turned out to be more of a problem. Three protein kinases were isolated from Candida utilis (Hemmings, 1978c; B. A. Hemmings, unpublished work). Comparison of their properties with the well-characterized protein kinases from mammalian tissues suggest that these kinases correspond to the catalytic subunit of the cyclic AMP-dependent protein kinase, casein kinase I and casein kinase II (Krebs & Beavo, 1979; Hathaway & Traugh, 1979). Although three kinases were found to phosphorylate the GDH to varying extents, they did not, under the conditions used, promote any loss of enzyme activity.

The rate and extent of phosphorylation observed with yeast casein kinase I is greater than that observed with the other two kinases. This reaction was selected for further study to try to resolve this paradox. Yeast casein kinase I gave incorporation of up to 0.8 mol of phosphate/mol of subunit of dephospho-GDH without any loss of enzyme activity, whereas phospho-GDH was not a substrate for the casein kinase I. This result appears to suggest that the kinase phosphorylated the GDH on the correct site and a 64500-mol. wt. fragment. Under conditions where only limited proteolysis occurs, the phospho-GDH could be completely re-activated; the activity of the dephospho-GDH was unaffected under similar conditions. Analysis of the products of limited proteolysis by using 32P-labelled GDH revealed that the phosphate group was located exclusively on the 48000-mol. wt. domain. Similar studies using limited proteolysis to probe the structure of NAD-GDH from Neurospora crassa have been carried out by Haberland et al. (1980). They also found that GDH could be cleaved into two domains, of mol. wts. 63000 and 47000, corresponding to an N-terminal and C-terminal portion of the enzyme respectively. Interestingly, the 63000-mol. wt. fragment contained considerable sequence homology to bovine NADP-GDH (mol. wt. of the subunit of this enzyme is 55000) and is thought to represent the catalytic domain. The 47000-mol. wt. fragment thus represents an N-terminal extension of the molecule and also appears to contain the phosphorylation site. Obviously the phosphorylation site must interact with the catalytic site on the 63000-mol. wt. fragment to cause a loss of enzyme activity.

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serine residue and not at a second site. In an attempt to ascertain whether this assumption was correct, peptide mapping was carried out by the method of Cleveland et al. (1977). The phosphopeptides derived from GDH labelled in both \textit{in vivo} and \textit{in vitro} by proteolysis with trypsin, chymotrypsin and \textit{Staphylococcus aureus} proteinase were detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography. In all cases the phosphopeptides from the enzyme labelled \textit{in vitro} had electrophoretic mobilities identical with those derived from the GDH labelled \textit{in vivo}.

This result suggests two possibilities, (a) that casein kinase I phosphorylates a serine residue located near to the serine residue labelled \textit{in vivo}, or (b) that casein kinase I phosphorylates the authentic serine residue but the isolated GDH is ‘frozen’ in an active conformation.

\textbf{Inactivation of NADP-dependent glutamate dehydrogenase}

During carbon starvation or transfer of yeast cultures to growth on amino acids as sole nitrogen, carbon and energy source, the NADP-GDH is inactivated (Hemmings, 1978a; Mazon, 1978). Immunochemical and genetical techniques have been used to establish the mechanism of inactivation (Hemmings, 1978a; Mazon & Hemmings, 1979; Hemmings \textit{et al.}, 1980).

The inactivation of NADP-GDH is slow (half-life \(t_1 = 60-120\text{ min}\)) compared with that of the NADH-GDH; it is also energy-dependent and irreversible (Hemmings, 1978a; Mazon, 1978). Experiments using cycloheximide demonstrated that the reappearance of NADP-GDH activity in yeast cultures previously starved for carbon was totally dependent on protein synthesis \textit{de novo}.

Immunochemical determination of the NADP-GDH demonstrated that the amount of precipitable antigenic material paralleled the loss of enzyme activity during inactivation. It should be pointed out that during normal exponential growth the rate of degradation of NADP-GDH is very low, about 5\%\text{ generation}. Thus it appears that at the onset of carbon starvation the system for proteolysis GDH is activated. Experiments were carried out to identify the components of this system by using a genetical approach.

Preliminary experiments suggested that proteinase B (an endo serine proteinase) is responsible for enzyme inactivation (Hemmings & Mazon, 1979). To test this possibility, the inactivation of GDH was examined in several proteinase-deficient mutants of \textit{S. cerevisiae} (Hemmings \textit{et al.}, 1980). Strains bearing mutations in the structural gene for proteinase B, proteinase C (a carboxypeptidase) or in both genes catalysed the inactivation and initial proteolytic cleavage of NADP-GDH at a rate indistinguishable from that of the wild type. In addition the pleiotropic mutation, \textit{pp} \text{e} 4-3, which results in a deficiency for proteinase A (an acid proteinase) as well as proteinases B and C, did not affect the inactivation or initial proteolytic cleavage.

These results suggest that these proteinases, all of which are found in the vacuole, do not play any significant role in the initial proteolytic cleavage of GDH. The evidence does not preclude a role for the vacuolar proteinases in any degradation that might occur subsequent to the initial modification associated with the loss of activity and immunoreactivity.

It is therefore postulated that there should be another proteolytic system probably in the cytosol, which is responsible for specific enzyme degradation.