AMP-ACTIVATION OF AN ALLOSTERIC NAD -DEPENDENT GLUTAMATE DEHYDROGENASE

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In most bacteria, higher plants and animals, only one glutamate dehydrogenase is known to be present (Sanwal and Lata, 1964). This enzyme may be specific for either NADP or NAD or non-specific as in the well-studied case of beef-liver glutamate dehydrogenase. However, in the facultative chemoautotroph, Thiobacillus novellus, two glutamate dehydrogenases have been found, one specific for NADP, and another for NAD (LéJohn, 1967). A similar situation has been reported for Neurospora (Sanwal and Lata, 1961), yeast (Holzer, 1957) Schizophyllum (Dennen and Neiderpreum, 1967). In all cases studied, the NAD-dependent glutamate dehydrogenase has been shown to be inducible. It becomes very important then to determine the physiological reason why there should be two of these enzymes, with distinct coenzyme specificities, catalyzing the same reaction which one could conceivably achieve.

The enzymes isolated from T. novellus were especially amenable to in vitro studies that may provide an answer to what has been suspected for some time, viz., that the NAD-dependent enzyme acts in a purely catabolic manner. This communication reports the influence of AMP as an allosteric effector of the NAD-dependent glutamate dehydrogenase altering

the equilibrium constants so as to favour glutamate breakdown. The effect is quite specific for AMP among a variety of nucleotides tested. ATP, apparently, reverses the AMP effect.

MATERIALS AND METHODS

The NAD- and NADP- dependent glutamate dehydrogenases were purified from cultures derived from a single cell isolate of Thiobacillus novellus (ATCC 8093). Growth and other conditions for induction of the NAD-dependent enzyme will be published separately.

Purification of Glutamate Dehydrogenase. Bacteria were harvested by centrifugation, washed twice in 0.01 M Tris-HCl, pH 8, and resuspended in 5 vol 0.05 M Tris-HCl, pH 8, containing 10^{-3} M GSH (TG) buffer. When necessary, AMP was included at all stages of the isolation procedure at 10^{-4} M concentration. The cells were disrupted in a 10 KC Raytheon sonic disintegrator for 30 min at 5°C. Cell debris was removed by centrifugation at 48,100 x g for 15 min at 2°C. The supernatant solution was treated with DNAse (50µg/ml), RNAse (50µg/ml) at 37°C for 30 min, rapidly brought to 55°C within 2 min and held at that temperature for 5 min, then rapidly cooled to 5°C. The thick viscous extract was made to 2 vol with 0.05 M Tris-HCl, pH 8, centrifuged at 48,100 x g for 20 min at 2°C. The supernate was carefully siphoned out from the denatured proteins and adsorbed onto a column of DEAE-cellulose (2.5 x 30 cm) previously equilibrated with 0.05 M TG buffer, pH 7.5. The enzymes were eluted off as separate peaks, with some overlap, using a linear gradient of 0.05 M - 0.5 M TG, pH 7.5 buffer. Further purification was achieved by differential precipitation with ammonium sulphate (45% saturation for NAD-dependent enzyme;

and 65% saturation for the NADP-dependent enzyme) and rechromatography on DEAE-cellulose using the same gradient at pH 8. Both enzymes were purified over 250-fold.

Enzyme Assays. A Gilford Model 2000 recording spectrophotometer was used for initial velocity measurements at 340 mμ. The enzymes were assayed according to the procedure of Sanwal and Lata, (1964). The NAD-dependent enzyme was easily desensitized to AMP activation by repeated freezing and thawing. Consequently, all assays were conducted within 48 hours of enzyme purification, or alternatively, the enzyme was stored in suitable aliquots at -20°C, thawed and used only once when required.

EXPERIMENTS AND DISCUSSION

The NAD-dependent and NADP-dependent enzymes both catalyzed the reversible formation of α-ketoglutarate from glutamate. Normal Michaelis-Menten kinetics indicated that the equilibrium constants of the NAD-dependent enzyme favoured the formation of glutamate (see K_m values in Table I) in vitro, in the absence

TABLE I

Michaelis-constants of NAD-dependent glutamate dehydrogenase with L-glutamate, α-ketoglutarate, oxidized and reduced NAD, and ammonia in the presence and absence of AMP

Substrate	K_m values in moles/litre (+ AMP)*	(- AMP)
L-Glutamate	1.1×10^{-3}	2.5×10^{-3}
NAD	1.4×10^{-4}	2.0×10^{-4}
NADH	8.0×10^{-5}	4.4×10^{-6}
α-Ketoglutarate	1.0×10^{-2}	4.0×10^{-4}
Ammonia	2.3×10^{-2}	5.0×10^{-3}

* AMP at a fixed concentration of 10^{-3} M.

of AMP. AMP modified the K_m values so as to favour glutamate breakdown. The reaction kinetics of the NADP-dependent enzyme were unaffected by AMP. Consequently, data in this report will be restricted to the NAD-dependent enzyme.

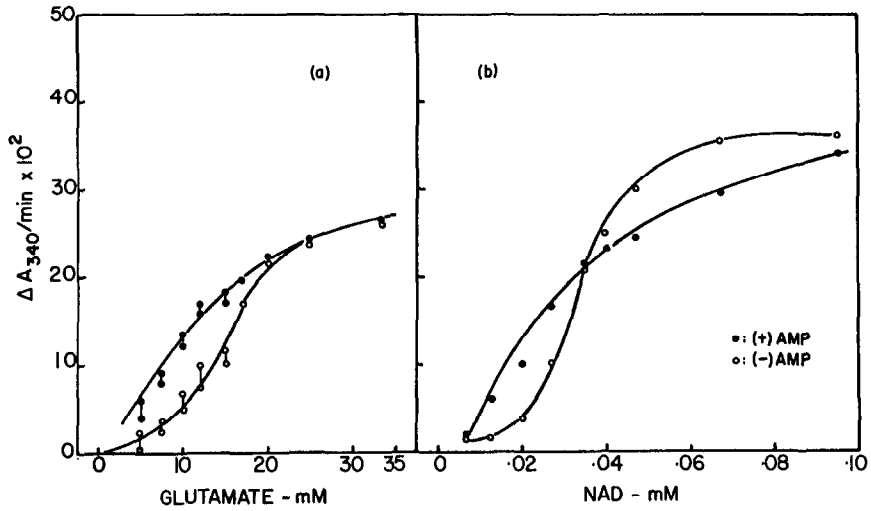

Fig. 1(a). Effects of AMP on the kinetics of NAD-dependent glutamate dehydrogenase during oxidative deamination of glutamate to α-ketoglutarate. The reaction mixture contained 0.2 M Tris-HCl, pH 9.5; 1 mM AMP where indicated; and a fixed concentration of 0.05 mM NAD. (b). The reaction mixture was the same as for (a) except that Glutamate was fixed at 16 mM and NAD varied.

When standard reaction velocity vs. substrate plots were made on the NAD-dependent enzyme, it was observed that the curves were clearly sigmoidal in the direction of glutamate breakdown (Fig. 1 a & b), and hyperbolic in the direction of glutamate synthesis (Fig. 2 a & b). The sigmoidal curve could be modified into hyperbolic plots when extremely high, and non-physiological concentrations of substrates were used

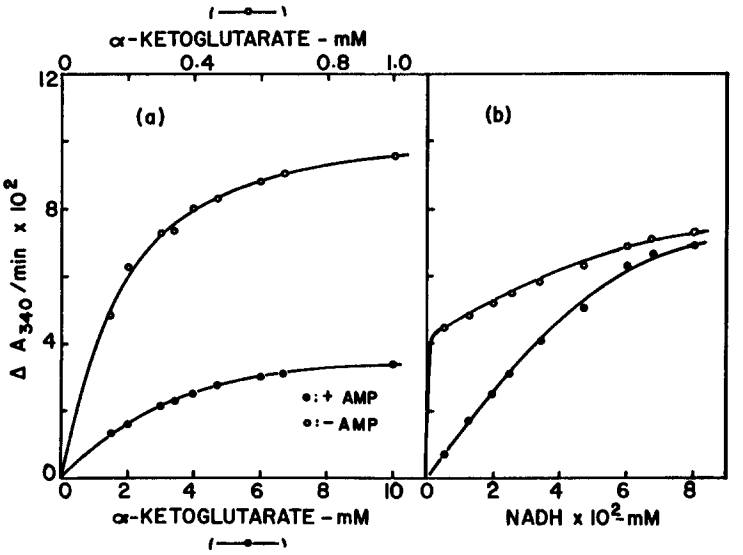

Fig. 2(a). Effect of AMP on the kinetics of NAD-dependent glutamate dehydrogenase during the reductive amination of α-ketoglutarate. Reaction mixture contained 0.2 M Tris-HCl, pH 8.0; 0.02 mM NADH; 40 mM $(NH_4)_2SO_4$; and 1 mM AMP where indicated. α-ketoglutarate as varied substrate. (b). The reaction mixture was the same as for (a) except that α-ketoglutarate was fixed at 1.67 mM and NADH was varied.

(25 - 50 mM glutamate, and 0.2 mM NAD).

AMP-Effect. Attempts were then made to find a suitable effector that could convert the observed sigmoidal plots into hyperbolic curves at physiological concentrations. Among a variety of nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, ITP, IDP, IMP) and macroions (poly-lysine, spermidine, polyadenine, and protamine) only AMP and to a lesser extent, ADP, were active as effectors. The activation effect was optimal at 10^{-3} M - 10^{-4} M. The change from sigmoidal to hyperbolic curves with AMP as effector is shown in Fig. 1 a & b. When AMP was used in the reverse reaction of glutamate formation from α-ketoglutarate, AMP was inhibitory (Fig. 2 a & b) with a correspondingly

large increase in K_m for all the substrates involved (Table I).

ATP-Effect. It was a logical thesis to suppose that ATP may act in the opposite manner to AMP as an effector. The results of a typical experiment in which ATP, ADP, and AMP act in an opposing manner are shown in the Lineweaver-Burke plots of Fig. 3. ATP slightly activated the formation of glutamate from α-ketoglutarate while ADP and AMP inhibited the same reaction at low substrate concentrations. When tested in the reverse direction, ATP failed to inhibit the breakdown of glutamate. A theory is that the ratio of ATP/AMP may be critical in this regulation. Further work is being done on this aspect.

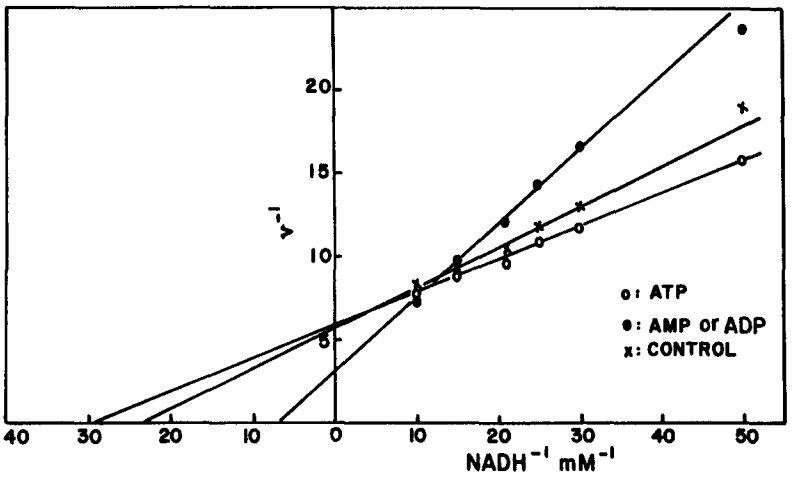

Fig. 3. Rate of reductive amination of α-ketoglutarate as a function of NADH concentration with ATP, ADP, and AMP as effectors. Reaction mixture contained 0.2M Tris-HCl, pH 8.0; 6.67 mM α-ketoglutarate; 40 mM $(NH_4)_2SO_4$; and one of ATP, ADP and AMP at a concentration of 1 mM. NADH as varied substrate.

These investigations point to a probable metabolic regulation of the NAD-dependent glutamate dehydrogenase activity by AMP and ATP in T. novellus. This enzyme, seemingly, functions in a purely catabolic manner supplying ATP during the

breakdown of glutamate. Breakdown of ATP into AMP and ADP would provide the necessary effectors (ADP, AMP) that activate the enzyme to a state that ensures continuous breakdown of glutamate. When high levels of ATP accumulate, the equilibrium may be shifted slightly so as to favour glutamate accumulation. A delicate balance of ATP/AMP ratio could then regulate the activity of this enzyme. Indirect evidence supporting the concept that the NAD-dependent enzyme may function in a largely catabolic manner comes from studies which have shown that this enzyme becomes induced when the organism is forced to rely on glutamate as its sole carbon and energy source (LéJohn 1967). This may prove to be a very sophisticated regulation for enzymes that are involved in energy synthesis.

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