DEMONSTRATION OF AN EFFECTOR SITE FOR THE ENZYME INOSINE 5'-PHOSPHATE DEHYDROGENASE*,**

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According to current thought, a regulatory enzyme, often following the branch point in a bifurcated pathway, generally can be expected to possess at least two unique features: (1) the reaction catalyzed should be essentially irreversible, and (2) the order of the reaction can be expected to be greater than one with respect to substrate and/or regulatory metabolites (i.e., feed-back inhibitors). The enzyme inosine 5'-phosphate dehydrogenase (IMP-DH; IMP:NAD oxidoreductase; EC 1.2.1.14) from enteric bacteria is the first enzyme in the purine anabolic pathway unique to guanylic acid (GMP) biosynthesis. The detailed reaction catalyzed by the enzyme was examined by Magasanik, Moyed and Gehring (1957), who showed that the reaction was irreversible over the pH range 5.5 to 8.8. Data appearing in a later paper by Mager and Magasanik (1960), showing the inhibition of IMP-DH by GMP, can be replotted by the method of Dixon (1/V vs. I) to reveal a nonlinear response of the inhibition to increasing concentrations of GMP.

These observations are consistent with the idea that IMP-DH may act as a regulatory enzyme. Recently however, Hampton and Nomura (1967) have presented evidence that IMP-DH is inhibited in a purely competitive

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fashion by GMP, and does not exhibit any 'allosteric' properties. Working with partially purified cell-free extracts of Salmonella typhimurium, we can show that there is a GMP recognition site on the IMP-DH molecule which is distinct from the active site for IMP. The present communication reports this evidence and further describes the action of GMP as a feed-back inhibitor eliciting 'allosteric' kinetics of the IMP-DH enzyme.

Careful handling of IMP-DH from Salmonella was found to be important in maintaining kinetic integrity. The enzyme preparation used in this report was frozen and thawed no more than two times during a given experiment. Extracts were prepared from fully derepressed cultures of the guanine auxotroph strain Gua-1 of Salmonella typhimurium grown at 37° in a minimal salts medium containing limiting concentrations of guanine. Following harvesting,washing and resuspending in sodium phosphate buffer (0.05 M, pH 7.5) at a concentration of 1 gm cell paste / 9 ml of buffer, the ice-cold cell suspensions were sonicated (Branson model 110s) at optimal power for no longer than two 45-second bursts. The cell suspension was kept in an ice-water bath during sonication and cooled an additional 30 seconds between bursts. The sonicate was centrifuged at 4° at 30,000xg and the supernatant treated with solid ammonium sulfate at 4°C. The fraction precipitating between 0% and 30% saturation was carefully brought into solution in a minimal volume of buffer, filtered through a Sephadex G-25 column equilibrated with the same phosphate buffer at room temperature, then assayed and frozen.

We have chosen the zwiterion TES described by Good et al. (1966) for use as a buffer in this series of experiments. With this buffer, in the assay system described by Magasanik, Moyed and Gehring, we have determined the K_m value for IMP as a function of pH over the range of 7.3 to 8.5. We simultaneously determined K_i values for GMP at a constant GMP concentration of 0.2 mM. The data are plotted in Figure 1 as pK_m (A) and pK_i (B) vs. pH. Dixon and Webb (1964) have shown that in plots

of this type, inflections correspond to ionizations within the system and that the intersections of the straight line segments give the pKs of these ionizing groups. Over the pH range of 7.5 to 8.3 the pK_m is

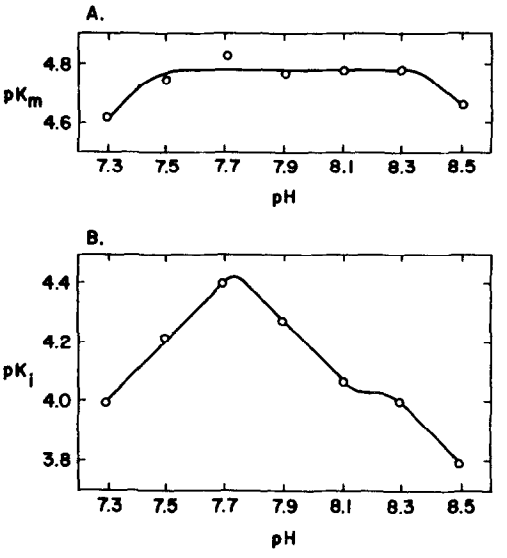

Figure 1. Effect of pH on Kinetic Constants of IMP-DH. Each assay contained TES-HCl, 100 mM; DPN, 2.5 mM; KCl, 10 mM; reduced glutathione, 3.5 mM; GMP when present, 0.2 mM; and protein, 9.75 μgm in a total reaction volume of 0.5 ml. The reaction was started by the addition of IMP to a final concentration of 0.05 mM to 0.4 mM and the reaction rate followed (Gilford model 2000) to no more than 10% of completion at 340 mμ. A) pK_m = negative logarithm of the K_m which was determined from a double reciprocal plot. B) pK_i = negative logarithm of K_i, determined from the slope ratios of the same plot using the linear portion of the line generated in the presence of GMP.

essentially constant (Part A). There appears to be, at the pH extremes tested, an ionization of a group involved in the formation of the E-S complex -- either in the free enzyme or in the free substrate. Since IMP has no ionizing group with a pK near the inflection point at pH 8.3, the ionization is that of a group on the enzyme presumably at or near the active site.

If pK_m and pK_i predominantly represent a binding process, and neither IMP nor GMP possess groups ionizing over the pH range 7.0 - 8.5, then the pK vs. pH functions would be expected to be identical for compounds binding at a common site. This would be the case for a com-

petitive inhibitor. As can be seen from Part B of Figure 1, this is apparently not so for the case of GMP. Since neither GMP nor IMP has groups which ionize in the pH region of 7.7, the inflection point in the graph, we interpret these results to suggest that there is a GMP recognition site on the enzyme which has a distinct pK and that this site differs from the IMP recognition site.

In a recent review, Cleland (1967) has pointed out that, for the catalytic potential of an enzyme to be fully utilized, the kinetic parameters of the enzyme must be within the range of the intracellular concentrations of the relevant substrate or effector molecules. From the work of Smith and Maaløe (1964) on pool levels of nucleotides, and from the determinations by Roberts et al. (1955) on cell volumes, we have calculated that the intracellular concentration of GMP probably does not exceed 0.4 mM. It is equally probable that the intracellular concentration of IMP will be in the region of the K_m for IMP-DH, around 0.02 mM for IMP.

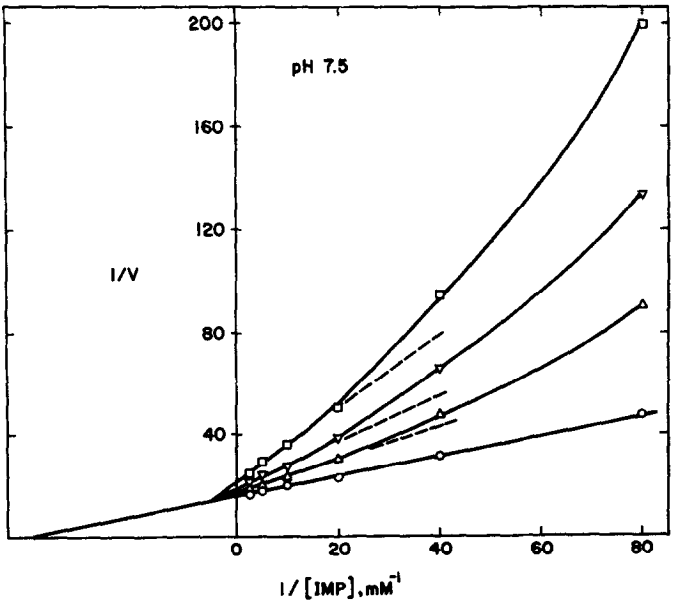

Figure 2. The Effect of GMP on Double-Reciprocal Plots of IMP-DH Activity. Assay conditions are as in Figure 1 at pH 7.5. ○, no GMP; △, GMP = 0.05 mM; ▽, GMP = 0.1 mM; □, GMP = 0.2 mM.

With this in mind, the inhibition exhibited by GMP at low concentrations of IMP was examined at pH 7.5. The data appear in Figure 2. A non-linear relationship can be seen at IMP concentrations between 0.0125 mM and 0.1 mM. When the data are replotted according to Atkinson et al. (1965), an interaction factor n can be determined at each concentration of the effector, GMP. In this case, at GMP = 0.05 mM, n = 1.13. When GMP = 0.1 mM, n = 1.18 and at GMP = 0.2 mM, n = 1.30. Interaction factors approaching 2.0 have been seen in extracts from "wild type" strains described by Levin (1967) and will be the subject of a future report. The factor n is considered to be a function of the number of substrate binding sites and the degree of interaction between them; the possession of n values greater than one is usually considered to be important for a regulatory or allosteric enzyme. Our observations on the feed-back regulation elicited by GMP on IMP-DH are consistent with the generalization (Monod et al., 1963) that a regulatory enzyme will possess 'allosteric' kinetics in the presence of a pathway end-product.

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