EQUILIBRIUM PROTECTION STUDIES OF THE INTERACTION OF BOVINE GLUTAMATE DEHYDROGENASE WITH PURINE NUCLEOTIDE EFFECTORS

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1. Introduction

Bovine liver glutamate dehydrogenase (GDH) is an allosteric enzyme [1–4]. Although its heterotropic effectors, ADP and GTP, are structurally related to the coenzymes and homotropic effectors, $NAD(P)^+$ and NAD(P)H, there is now abundant evidence that the enzyme possesses separate, regulatory nucleotide sites in addition to the catalytic sites [2,5–10]. It is still not entirely clear, however, to what extent these sites overlap.

In recent studies [11–15] of reversible enzyme inactivation by pyridoxal 5′-phospate (PLP) we have developed a technique which we term 'equilibrium protection'. Even at saturating concentrations, PLP causes only partial inactivation of GDH and other dehydrogenases [12–16], because, although in each case the covalently-modified enzyme (Schiff base) is completely inactive, it exists in equilibrium with a readily dissociable, non-covalent enzyme–PLP complex. The residual activity at equilibrium may be measured very precisely with small amounts of enzyme, and perturbation of the equilibrium thus provides a sensitive means of monitoring ligand binding and consequent conformational changes.

Success in detecting a conformational change in GDH following the binding of the substrate, 2-oxoglutarate, or the substrate analogue, glutarate [11], prompted us to try the equilibrium protection method in studying the interactions of ADP and GTP with the enzyme.

2. Experimental

GDH, coenzymes and nucleotides were obtained from Boehringer Corp. (London) Ltd.

For inactivation experiments, GDH (0.25 mg/ml) was incubated at 25°C in test tubes protected from the light with metal foil. Reactions were carried out in 0.1 M K-phosphate, pH 7 or 8, containing 1.8 mM PLP and protecting agents at the concentrations indicated in the text. Samples (2–5 μl) were withdrawn for fluorimetric assay of catalytic activity in a reaction mixture containing 5 mM glutamate, 0.6 mM NAD^+ and 0.11 M Na-phosphate, pH 7 or 8 according to the inactivation conditions.

Other procedures were as described previously [11].

3. Results

Fig.1 shows that, at pH7, in the presence of 1.8 mM PLP, the activity of GDH declines to 10% of the initial value. NADH at a saturating concentration (1 mM) gives only partial protection (residual activity, R.A., at equilibrium = 24%), in agreement with earlier work [17,12]. GTP and ADP (both 1 mM), either alone (fig.1) or together (not shown), are without effect on the course of inactivation (R.A. = 10%). In the presence of NADH, however, as previously noted by Goldin and Frieden [18], GTP considerably enhances the protection (fig.1): the enzyme was still over 60% active after 2½ h under these conditions. Detailed analysis shows, that, at pH 7, in the presence of NADH, GTP mainly affects the rate of inactivation rather than its final extent (fig.2); evidently the nucleotide slows down inactivation and reactivation equally.

Even in the presence of NADH, ADP is without effect (R.A. = 24%) (fig.1), but this nucleotide nevertheless completely nullifies the protective effect of

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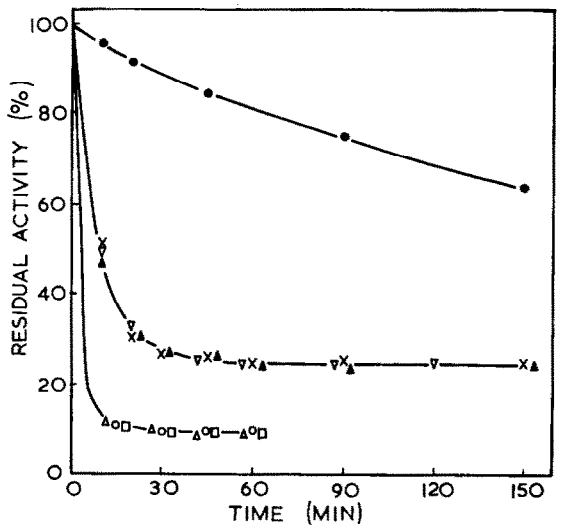

Fig.1. Effects of nucleotides on the inactivation of GDH by PLP at pH 7. GDH (0.25 mg/ml) was incubated at 25°C in 0.1 M K-phosphate buffer, pH 7, containing 1.8 mM PLP and the following additions: none (○), 1 mM ADP (△), 1 mM GTP (□), 1 mM NADH (▽), 1 mM NADH and 1 mM ADP (▲), 1 mM NADH and 1 mM GTP (●), NADH, ADP and GTP (all 1 mM) (×).

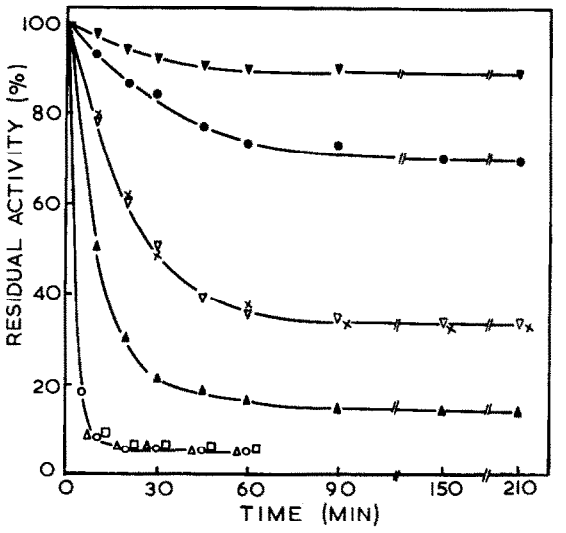

Fig.3. Effects of nucleotides on the inactivation of GDH by PLP at pH 8. GDH (0.25 mg/ml) was incubated at 25°C in 0.1 M K-phosphate, pH 8, containing 1.8 mM PLP and the following additions: none (○), 1 mM ADP (△), 1 mM GTP (□), 1 mM NADH (▽), 1 mM NADH and 1 mM ADP (▲), 1 mM NADH and 1 mM GTP (▼), 1 mM NADH and 0.1 mM GTP (●), NADH, ADP and GTP (all 1 mM) (×).

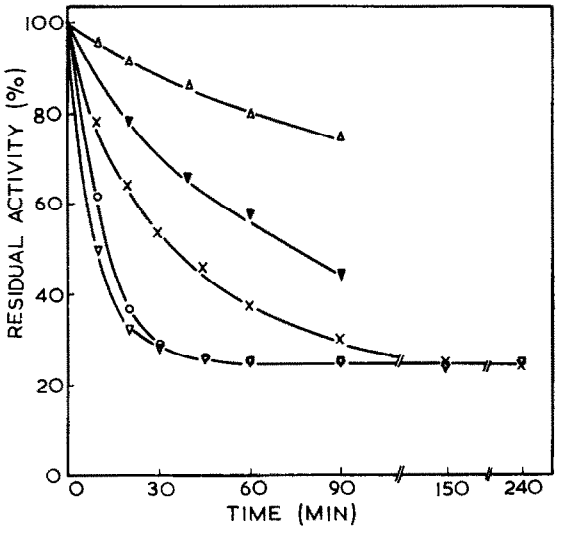

Fig.2. Effect of GTP on the inactivation of GDH by PLP in the presence of NADH at pH 7. Conditions as for fig.1 except for nucleotide additions as follows: 1 mM NADH in all incubations; GTP concentrations 0.5 mM (△), 0.2 mM (▼), 0.1 mM (×), 0.05 mM (○), 0 mM (▽).

GTP in an incubation with NADH, ADP and GTP (R.A. = 24%).

The regulatory properties of GDH are pH-dependent. Thus, ADP, which is an inhibitor at pH 7 [19, 20], is an activator at pH 8, whereas GTP is inhibitory at both pH values. It was therefore of interest to compare patterns of nucleotide protection at these pH values. Fig.3 shows that, at pH 8, GTP and ADP are again ineffective as agents of protection in the absence of NADH. In its presence they both affect the inactivation equilibrium, but in opposite senses. GTP confers additional protection, and the effect at pH 8 is clearly on the position of equilibrium (with saturating GTP R.A. = 88%), and not only, as at pH 7, on the rate of approach to that equilibrium. ADP, by contrast, partially reverses the protection given by NADH alone (figs.3,4). Residual activity was 34% in the presence of NADH as protecting agent, but only 15% when 1 mM ADP, sufficient to exert a maximal effect, was also present. The corresponding figure in the absence of protecting agents was 6%.

In the presence of GTP and NADH (1 mM), at pH

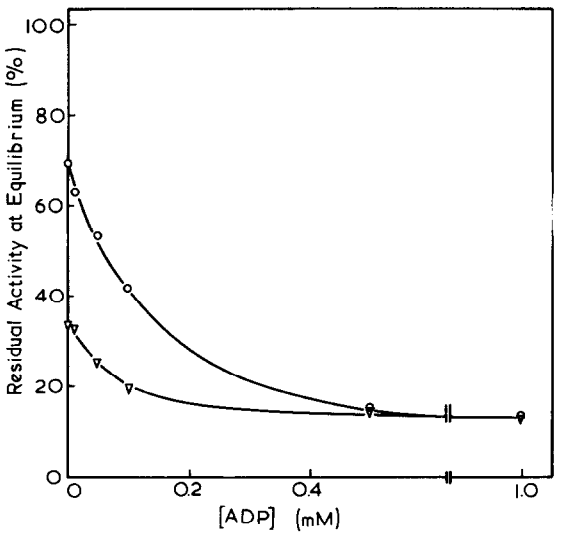

Fig.4. Effect of ADP on the residual activity of GDH inactivated by PLP in the presence of NADH ± GTP at pH 8. Each point represents the activity measured at equilibrium in an incubation under the conditions of fig.3. 1 mM NADH was present in all cases; GTP either 0.1 mM (○) or absent (Δ).

8 as at pH 7, the addition of ADP reversed the GTP effect (fig.3). When a sub-saturating GTP concentration (0.1 mM) was used, the residual activity at equilibrium was 68% in the absence of ADP, but only 15% when ADP was present at a saturating concentration (1 mM) (fig.4). The fact that this is exactly the same as the residual activity achieved in the absence of GTP (fig.4) suggests that ADP and GTP are mutually exclusive in their interactions with GDH.

It has been shown [11] that NAD^+ only protects GDH against PLP in the presence of dicarboxylic acid substrates or analogues. It seemed possible that the same dicarboxylates might enable ADP or GTP to influence the inactivation equilibrium in the absence of NADH. GDH was therefore incubated at pH 7 with PLP, 40 mM 2-oxoglutarate, and either GTP (1 mM) or ADP (1 mM). The protection observed was the same as that given by 2-oxoglutarate alone. Clearly the influence of the purine nucleotides on the course of inactivation is entirely dependent on the presence of coenzyme. Experiments less detailed than those shown in figs 1–4 indicated that NADPH is as effective as NADH in rendering the equilibrium between GDH and PLP reponsive to ADP and GTP.

4. Discussion

GTP and ADP can be bound by GDH even in the absence of coenzyme [21,22]. The fact that these nucleotides only affect the GDH–PLP equilibrium in the presence of coenzyme and that, at pH 8, ADP actually increases the extent of inactivation suggests that lysine 126, the site of modification by PLP [23], does not lie within the ADP or GTP sites. Direct binding studies have also shown that chemical modification of lysine 126 does not abolish binding of GTP [24] or ADP [25].

The enhancement of protection by GTP in the presence of NAD(P)H may be attributed to a conformational change in the protein. Other evidence for such a change has come from optical rotatory dispersion measurements [26], from studies with a fluorescent reporter group [27,28], and from chemical modification with acetic anhydride [29]: Colman and Frieden found that the presence of NADH and GTP increased the number of amino groups acetylated by this reagent.

The present results are in striking agreement with those of Colman and Frieden [30] in several respects, even though acetic anhydride is a much less selective reagent than PLP. Thus, at pH 7.15, GTP gave no protection in the absence of NADH, but strong protection in its presence; ADP was ineffective even with NADH present, but nevertheless counteracted the protective effect of GTP. These parallel results suggest that the essential residue modified by acetic anhydride may also be lysine 126. The agreement is remarkable since phosphate buffer was used in our experiments, whereas Colman and Frieden used Tris, in which the stability [31,32], kinetic properties [32] and sulphydryl reactivity [33] of GDH are markedly altered.

The most important results from the present study are those relating to the antagonism between ADP and GTP. At both pH 7 and pH 8, in the presence of NADH, ADP completely reverses the protective effect of GTP. The situation is clearest, however, at pH 8, since, at pH 8, ADP decreases the effectiveness of protection by NADH even in the absence of GTP. It is thus possible to show (fig.4) that, in the presence of NADH and GTP, saturation with ADP changes the position of the inactivation equilibrium to that which would be obtained with ADP plus NADH rather than with NADH alone. The simplest explanation of this clearcut observation is that ADP displaces GTP, either

because they bind at the same locus on the enzyme, or because the two nucleotides are bound by the enzyme in two different conformational states. Such an explanation would be consistent with kinetic results [34,35] showing that ADP and GTP are mutually exclusive in their effects on the rate of the enzyme-catalysed reaction. It conflicts, however, with the evidence of di Prisco [6] suggesting, also on the basis of protection studies, that GDH may be simultaneously saturated with NAD^+ at the catalytic site, and GTP and ADP at their respective, separate sites. It conflicts also with the detailed binding studies of Koberstein and Sund [7], which show that there are two NADH sites per subunit, one of them being a regulatory site also available for ADP binding, and a third site for GTP, which may be filled without interfering with binding of NADH to the other two sites. The only interpretation consistent with all of these results is that there are indeed three sites per subunit, but that occupation of the ADP site totally reverses the conformational change brought about by GTP in the presence of NADH. Experiments are in progress designed to test this point further.

It is difficult to believe that such a complex control mechanism can be without biological significance, and yet no satisfactory account has yet been given of the metabolic significance of the allosteric properties of GDH. Is GDH in mammalian liver so active that it equilibrates its reactants under all physiological conditions [36], or are there [37] conditions under which this reaction becomes rate-limiting and therefore perhaps subject to effective allosteric control? Another possibility that should be borne in mind is that the control properties of this enzyme may be relevant to its function in some tissue other than liver.

Acknowledgements

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