Mode of Interaction of Purine Nucleotides and Amino Acids with Glutamate Dehydrogenase

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1. The mode of action of purine nucleotides and amino acids on the activity of ox-liver glutamate dehydrogenase was investigated. 2. The addition of two chemically unrelated activators, at concentrations below saturation levels, enhanced the enzyme activity much more than a twofold concentration of each one separately. No such synergistic activation was observed when a combination of two members of the same group was tested. 3. With saturating concentrations of the activators, the increase in enzymic activity produced by a pair of chemically related effectors was either identical with or even below that achieved by the more active effector. However, the combination of two unrelated activators, at saturating amounts, still yielded a higher enzyme activity than with each one singly. 4. Unlike ADP, L-leucine was incapable of overcoming completely the inhibition produced by GTP. 5. It is suggested that purine nucleotides and amino acids bind to separate group-specific allosteric sites of this enzyme. 6. The possible physiological significance of these findings with regard to the regulation of the cellular functions of this enzyme is discussed.

Several groups of chemically unrelated compounds can affect the activity of ox-liver glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase (deaminating), EC 1.4.1.3]. Though some essential amino acids activate the enzyme (Yielding & Tomkins, 1961), among the purine nucleotides both activators and inhibitors are found (Frieden, 1963). In addition, NADH (Frieden, 1959), steroid hormones (Yielding & Tomkins, 1960) and thyroxine (Wolff, 1962) also inhibit the enzymic activity. Further, these studies demonstrate that the above-mentioned substances bind at a site (or sites) other than the active site, and thus belong to the class of allosteric effectors (Monod, Changeux & Jacob, 1963). Little doubt exists at present that more than one allosteric site must be postulated for this enzyme. The number and specificity of the allosteric sites for some of the effectors have been considered by various authors (Frieden, 1963; Wolff, 1962; Tomkins, Yielding, Talal & Curran, 1963).

In the present study evidence is provided that strongly suggests that the purine nucleotides and amino acids bind to distinct group-specific allosteric sites. A preliminary account of this investigation has been presented (Hershko & Kindler, 1965).

MATERIALS AND METHODS

AMP, ADP, GTP and NADP⁺ were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. The various amino acids were purchased from Mann Research Labora-

tories Inc., New York, N.Y., U.S.A. Purity of the reagents was checked by paper chromatography.

Crystalline ox-liver glutamate dehydrogenase (C. F. Boehringer und Sohne G.m.b.H., Mannheim, Germany) was obtained in 50% glycerol at a concentration of 10 mg./ml. Before use, the enzyme was diluted 100-fold in 0.1 M-tris buffer, pH 7.4, containing KH₂PO₄ (1 mM) and EDTA (0.1 mM); in this solution the diluted enzyme was stable in ice for at least 6 hr.

Enzymic activity was measured by following the increase in *E*₃₄₀ with a Beckmann DU spectrophotometer at room temperature. In all the experiments the reaction mixture consisted of tris buffer, pH 8.0 (30 μmoles), L-glutamate (neutralized with tris) (20 μmoles), NADP⁺ (1 μmole) and the various compounds tested in a final volume of 1.0 ml. The concentrations of glutamate and NADP⁺ are saturating with respect to the enzyme. NADP⁺ was chosen because, unlike NAD⁺, it is not an activator at high concentration (Frieden, 1959). The reaction was initiated by adding 0.05 ml. of the diluted enzyme solution (containing 5 μg. of protein) to the complete reaction mixture. Initial velocities were obtained at all instances by following the reaction rate during the first minute. Each result presented is the mean value of at least two determinations.

RESULTS

To examine whether purine nucleotides and amino acids bind at the same site, different combinations of two activators were added to the reaction mixture. The concentration chosen for each effector was below its saturation level and gave about 40% increase in the enzyme activity. Table 1 shows that

Table 1. *Synergistic activation of glutamate dehydrogenase by adenine nucleotides and amino acids*

Enzymic activity was measured by the procedure described in the Materials and Methods section.

Additions	$\Delta E_{340}/\text{min.}$	Activation (%)
None	0.062	—
ADP (15 μM)	0.087	40
AMP (80 μM)	0.088	42
L-Leucine (10 mM)	0.090	45
L-Isoleucine (45 mM)	0.083	34
L-Methionine (90 mM)	0.084	35
ADP (30 μM)	0.094	51
AMP (160 μM)	0.093	50
L-Leucine (20 mM)	0.093	50
L-Isoleucine (90 mM)	0.089	44
L-Methionine (180 mM)	0.090	45
ADP (15 μM) + AMP (80 μM)	0.095	53
L-Leucine (10 mM) + L-isoleucine (45 mM)	0.091	47
L-Leucine (10 mM) + L-methionine (90 mM)	0.097	56
L-Isoleucine (45 mM) + L-methionine (90 mM)	0.091	47
ADP (15 μM) + L-leucine (10 mM)	0.118	90
ADP (15 μM) + L-isoleucine (45 mM)	0.118	90
ADP (15 μM) + L-methionine (90 mM)	0.116	87
AMP (80 μM) + L-leucine (10 mM)	0.112	81
AMP (80 μM) + L-isoleucine (45 mM)	0.112	81
AMP (80 μM) + L-methionine (90 mM)	0.113	82

the combined addition of two activators from the same chemical group (e.g. AMP with ADP, L-leucine with L-methionine etc.) produces an activation that is not higher than that achieved by a twofold concentration of each one separately (about 50% activation). On the other hand, the addition of two chemically unrelated activators (e.g. ADP with L-leucine, AMP with L-methionine etc.) enhances the enzyme activity in a synergistic manner to a far greater degree (about 80–90% activation) (Table 1).

One possible explanation of these findings is that adenine nucleotides and amino acids bind to separate sites of this enzyme. Thus two activators of the same group would compete on their common site, but two chemically unrelated activators will not interfere with their mutual binding to the enzyme. In the latter case, each activator would be able to exert its complete effect independently, thus bringing about a synergistic enhancement of the enzyme activity.

To test the validity of this suggestion, the influence of varying the concentration of an activating amino acid on the effects of ADP and L-leucine was studied. Norvaline was chosen, because of its high solubility; the concentrations of ADP and L-leucine used are identical with those used in the previous experiment. As illustrated in Fig. 1, on increasing the concentration of norvaline the additional activation by L-leucine is progressively diminished and vanishes at high concentrations of norvaline. On the other hand, a corresponding amount of ADP still causes an additional activation

Fig. 1. Effect of ADP and L-leucine on glutamate-dehydrogenase activity in the presence of DL-norvaline. Experimental conditions were as described in the Materials and Methods section. ○, Control; □, in the presence of L-leucine (10 mM); Δ, in the presence of ADP (15 μM).

even at high concentrations of norvaline. The above findings are in accord with the interpretation that norvaline displaces L-leucine from the 'amino acid site', but not ADP from the 'purine nucleotide site'.

The effect of the combined addition of saturating concentrations of the different activators is shown in Table 2. Each activator was used at about a tenfold higher concentration than that used in the previous experiments. Each activator singly gives a different degree of maximal activation. The combined

Table 2. Activation of glutamate dehydrogenase by combination of various activators at saturating concentrations

Enzymic activity was measured by the procedure described in the Materials and Methods section.

Additions	$\Delta E_{340}/\text{min.}$	Activation (%)
None	0.062	—
ADP (0.2 mM)	0.113	82
AMP (1 mM)	0.095	53
L-Leucine (0.1 M)	0.102	65
ADP (0.2 mM) + AMP (1 mM)	0.096	55
ADP (0.2 mM) + L-leucine (0.1 M)	0.133	115
AMP (1 mM) + L-leucine (0.1 M)	0.122	97

Fig. 2. Influence of ADP on the inhibition produced by GTP on the activity of glutamate dehydrogenase. Experimental conditions were as described in the Materials and Methods section. $\circ$, Control; in the presence of the following concentrations of GTP: $\triangle$, 1.5 μM ; $\square$, 3 μM ; $\bullet$, 10 μM .

addition of ADP with AMP yields less activation than ADP alone, which is compatible with the interpretation that ADP is partly displaced from its site by the high amount of AMP. Conversely, the supplementation of saturating concentrations of ADP with L-leucine or of AMP with L-leucine still elicits a higher enzyme activity than each one singly (Table 2).

The above conclusions were further substantiated by studying the mode of influence of ADP and L-leucine on the inhibition produced by GTP on glutamate-dehydrogenase activity (Figs. 2 and 3). Wolff (1962) has shown that GTP markedly inhibits this enzyme and that this inhibition is reversed by ADP. These findings are confirmed by our results presented in Fig. 2, which illustrates the complete reversal of the inhibition of GTP by ADP. Contrarily, L-leucine was unable to overcome completely the inhibition produced by similar amounts of GTP;

Fig. 3. Influence of L-leucine on the inhibition produced by GTP on the activity of glutamate dehydrogenase. Experimental conditions were as described in the Materials and Methods section. $\circ$, Control; in the presence of the following concentrations of GTP: $\triangle$, 1.5 μM ; $\square$, 3 μM ; $\bullet$, 10 μM .

rather, L-leucine restores the enzyme activity only to a finite value, which depends on the concentration of GTP (Fig. 3). These findings constitute evidence in favour of the conclusion that GTP is displaced from the 'purine nucleotide site' by ADP, but not by L-leucine, which binds to another site.

DISCUSSION

Frieden (1963) postulated that all purine nucleotide effectors, both activators and inhibitors, bind to a common 'purine nucleotide site'. However, it has not been investigated whether or not the amino acid activators also share the same site. The marked structural difference between nucleotides and amino acids made it seem very unlikely that these substances would bind to a common allosteric site, which is, as a rule, highly stereospecific (Monod *et al.* 1963). The results of our kinetic experiments favour the conclusion that amino acid effectors do not bind to the 'purine nucleotide site', and that they share a common 'amino acid site'. This is based on the demonstration of competition between effectors that are members of the same chemical group, and on the finding that no such phenomenon occurs between chemically unrelated effectors.

The possibility that purine nucleotides and amino acids may act on different isoenzymes in the enzyme preparation was considered. In that case the activation brought forth by the combination of two unrelated activators would be expected to be equal to the sum of the individual activations under all conditions. The results presented in Table 2 show that with saturating amount of the activators the enhancement in activity is considerably less than the sum of activations, thus ruling out the possible

presence of more than one isoenzyme in the crystalline preparation used. It appears therefore more reasonable to assume that these substances act on a common enzyme molecule at different sites. A maximal activation produced by one effector will diminish the extent of additional activation by a second unrelated activator, owing to an upper limit of activation that can be achieved.

The question arises whether purine nucleotides and amino acids, though binding to different allosteric sites, exert their action by promoting the same conformational change in the enzyme molecule, namely 'allosteric transition' (Monod *et al.* 1963). Obviously kinetic experiments alone cannot give a satisfactory answer to this question, and more work employing different techniques is needed to settle this problem.

The problem of the number of different allosteric sites of glutamate dehydrogenase has already been raised by Frieden (1963), who has shown that the 'second site' for NADH is not identical with the purine nucleotide site. Wolff (1962) suggested that thyroxine can interact with both these sites. Yielding & Tomkins (1961) found that the inhibition produced by steroid hormones and diethylstilboestrol can be reversed by L-leucine, but did not indicate a specific site of binding for these substances. It is not yet known whether or not these hormones can interact with the proposed 'amino acid site'.

Though the exact mode of the regulation of the activity of glutamate dehydrogenase by these metabolites *in vivo* is at present unknown, it seems worth while to consider the possible physiological significance of the existence of separate allosteric sites for purine nucleotides and amino acids. The location of glutamate dehydrogenase on the crossroad of carbohydrate and amino acid metabolism and the possible role of the effectors in physiological

regulation have been discussed in the literature (Frieden, 1963; Tomkins *et al.* 1963; Wolff, 1962). If one restricts the consideration of the action of glutamate dehydrogenase only to its functions in deamination and transdeamination (Braunstein, 1957; Meister, 1955), one can regard the activation by amino acids as an example of precursor activation of their catabolism. On the other hand, the additional activation by ADP would indicate that this catabolic function of the enzyme is controlled by the efficiency of oxidative phosphorylation. The catabolism of the amino acids would thus be controlled in a concerted manner (a) by their concentration in the mitochondria and (b) by the demand for ATP synthesis. Such a regulatory mechanism would need a certain degree of independence in the response to these different metabolic demands, which could be achieved by the existence of the separate control sites. We may thus observe here a further example of multivalent regulation, somewhat resembling that described by Woolfolk & Stadtman (1964) for bacterial glutamine synthetase.

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