Multivalent Regulation of Glutamic Dehydrogenases from Fungi

EFFECTS OF ADENYLATES, GUANYLATES, AND ACYL COENZYME A DERIVATIVES*

(Received for publication, May 19, 1970)

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SUMMARY

Guanylates (GTP, GDP, and GMP), short chain acyl-CoA derivatives; ATP, and ADP, were found to be allosteric activators of NAD-specific glutamic dehydrogenases isolated from a unique group of fungi, the Oomycetes. Long chain acyl-CoA derivatives and AMP were very strong inhibitors of the enzyme. Pythium glutamic dehydrogenase, in particular, was shown to be sensitive to control by energy charge.

A total of five activators have now been discovered. These are NADP+, P-enolpyruvate, short chain acyl-CoA derivatives, GTP, and ATP. These activators functioned cumulatively to antagonize the effects of the inhibitors, citrate, AMP, and long chain acyl-CoA derivatives.

The activators acted as unidirectional stimulants of the biosynthetic reaction, while some of them inhibited the catabolic reaction unidirectionally. These glutamic dehydrogenases are genetically controlled. They are subjected to marked catabolic repression and inducible by glutamate. The substrates (ammonia and 2-ketoglutarate) of the biosynthetic reaction are allosteric inhibitors. The activators modulated the enzyme against allosteric inhibition by its substrates.

This multivalent control has been explained as a mechanism by which the enzyme effects its biosynthetic and catabolic roles in amphibolic reactions of the citric acid cycle.

A remarkable correlation has been made between lysine biosynthesis and allosteric control mechanisms among members of the Phycomycetes. The distribution of the two pathways of lysine biosynthesis among these simple fungi parallels the only two different forms of allosteric controls of NAD-specific glutamic dehydrogenases elucidated for all major orders of the Phycomycetes studied to date.

One such metabolic link is the oxidation-reduction reaction catalyzed by glutamic dehydrogenase. Although NAD-specific glutamic dehydrogenase has been regarded as a catabolic enzyme, there would be times when it must perform a biosynthetic role; e.g. when the NADP-specific variety is absent in an organism. This enzyme should therefore respond to fluctuations in adenylate concentrations (energy charge) as defined by Atkinson.

In this communication, we report on the effects of adenylates, short and long chain fatty acid-CoA esters, and guanylates (GTP, GDP, and GMP) as modulators of the catalytic activity of NAD-specific glutamic dehydrogenases obtained from a specialized group of fungi, Oomycetes. Preliminary findings have shown that the enzyme is controlled both at the genetic and enzymatic levels. Synthesis of the enzyme is repressed by catabolites. NADP+, NADPH, and P-enolpyruvate activate the enzyme in an allosteric manner. The activators act cumulatively to antagonize the inhibitory effects of citrate, AMP, and long chain fatty acid-CoA esters.

Among regulatory enzymes, glutamic dehydrogenase is unique. Glutamic dehydrogenases isolated from microorganisms and higher animals display different allosteric effects. Evidently, this protein has been a sensitive target for the evolution of regulatory mechanisms.

MATERIALS AND METHODS

Methods used in the treatment of all the fungi for enzyme isolation have been reported elsewhere. Kinetic assays were carried out with a Gilford model 2400 recording spectrophotometer, equipped with temperature control unit at 25°C.

Chemicals—CoA, acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, succinyl-CoA, dephospho-CoA, palmitoyl-CoA, and oleyl-CoA were purchased from P-L Biochemicals. All other chemicals were obtained from Sigma.

RESULTS

Response to "Energy Charge"—The organisms Achlya sp. (1969), Saprolegnia parasitica, and Pythium debaryanum used as experimental material in these studies are taxonomically related in the phenetic sense. But the NAD-specific glutamic dehydrogenases isolated from each organism display differently different responses to some of the allosteric modifiers. For example, glutamic dehydrogenases from Achlya and Saprolegnia are relatively insensitive to modulation by adenylates. The oxidative deamination (catabolic) reaction, when catalyzed by Achlya and Saprolegnia glutamic dehydrogenases, is inhibited.
Multivalent Regulation of Glutamic Dehydrogenases

Vol. 245, No. 21

Fig. 1. Effects of adenine nucleotides on the (a) catabolic and (b) biosynthetic reaction of Achlya NAD-specific glutamic dehydrogenase. The reaction rates $v_0$ and $v_e$ represent activities in the presence and absence of an effector. Effectors used were ATP (O) and AMP (•). Assays were conducted at pH 8 with substrates at the following concentrations: a, 33.33 mM glutamate, 2.66 mM NAD+, 10μg of enzyme, 66.67 mM Tris-acetate; b, 200 mM NH₄⁺, 10 mM α-ketoglutarate, 0.167 mM NADH, 66.67 mM Tris-acetate, 4 μg of enzyme.

65% by AMP and 22% by ATP at 5 mM concentrations (Fig. 1a). The reductive amination (biosynthetic) reaction is unaffected by similar concentrations of AMP and ATP (Fig. 1b). On the other hand, glutamic dehydrogenase from Pythium was found to be extremely sensitive to the influence of adenylates. ATP functioned as a mild activator and AMP as a strong inhibitor of the biosynthetic and catabolic reactions (Fig. 2, a and b). In these experiments, addition of adenylate kinase was omitted. According to the formulations of Atkinson (3), the response of Pythium glutamic dehydrogenase to varied proportions of AMP, ADP and ATP in catalyzing the catabolic and biosynthetic reactions was of the energy-utilizing (U-) type. In the absence of ADP, as in these experiments, absolute energy charge values were not obtained. Our interest was to observe the general pattern of response of the enzyme to adenylates. Although ADP, like ATP, activates the enzyme slightly, the "energy charge" values given in Fig. 2, a and b, are only apparent.

One point of significance is the disproportionately large difference in adenylate concentrations required to control the biosynthetic and catabolic reactions. The catabolic reaction was completely inhibited by 1 mM AMP whereas the biosynthetic reaction required 6 mM AMP for effective control. Therefore, at other than equilibrium conditions, the adenylate control system would be operative unidirectionally as defined previously (9). Although the catabolic reaction may be completely inhibited at adenylate acid levels above 1 mM, the biosynthetic reaction would be operative, albeit, at a reduced efficiency. With ATP present, the difference would be more marked because the adenylate effect is a nonlinear function of the total adenylates, not of the single components.

Activators and Energy Charge—Pythium, Achlya, and Saproleg-
nia are among a few organisms shown to possess an NAD-specific glutamic dehydrogenase that is activated by NADP⁺, NADPH, and P-enolpyruvate (4). A linked metabolic reaction is catalyzed by an NADP-specific isocitric dehydrogenase. We speculated that these two enzymes may act cooperatively and function as a transhydrogenase system. Some transhydrogenases are energy-linked (10). It was of signal interest to find that Pythium glutamic dehydrogenase is particularly susceptible to adenylate control. Because a multitude of activators have now been found (see later in this report), all activators, singly and in diverse combinations, were analyzed for their capability to antagonize or interact with the adenine nucleotides. The results presented in Fig. 2a show that P-enolpyruvate and NADP⁺ are the only ligands that can, independently, release the enzyme from AMP inhibition. The other activators, GTP, CoA, and derivatives, were incapable of doing this even when combined.

![Figure 3](image-url)

**Fig. 3.** Rate-concentration plots for (a) NH₄⁺; (b) α-ketoglutarate as variable substrate in the biosynthetic reaction catalyzed by Pythium NAD-specific glutamic dehydrogenase. Reactants used were as follows: a, 6.67 mM α-ketoglutarate, 0.167 mM NADH, 66.67 mM Tris-acetate, pH 8, 2 μg of enzyme; b, 20 mM NH₄⁺ and other components as in a, except that α-ketoglutarate concentration was varied as indicated.

### Table I

Summary of response of Pythium and Achlya NAD-specific glutamic dehydrogenases to variety of allosteric modulators

| Modifier          | Pythium | Achlya |
|-------------------|---------|--------|
| Reductive amination reactions |         |        |
| NADP⁺             | +       | +      |
| NADPH             | +       | +      |
| P-Enolpyruvate    | +       | +      |
| GTP (GDP, GMP)    | +       | +°     |
| CoA derivatives (short chain)⁶ | +       | +°     |
| Palmitoyl-CoA     | -       | -      |
| Oleoyl-CoA        | -       | -      |
| ATP (ADP)         | -       | -      |
| AMP               | -       | -      |
| 3',5'-AMP         | 0°      | 0      |
| 3',5'-GMP         | 0°      | 0      |
| Citrate (Isocitrate) | -      | -      |
| Dephospho-CoA     | 0       | 0      |

| Oxidative deamination reactions |      | +      |
| NADP⁺             | 0°     | +      |
| P-Enolpyruvate    | -      | -      |
| GTP (GDP, GMP)    | 0      | -      |
| CoA derivatives    | -      | -      |
| Palmitoyl-CoA     | -      | -      |
| ATP (ADP)         | -      | -      |
| AMP               | -      | -      |
| Citrate           | -      | -      |

- Slight activating effect.
- CoA; acetyl-CoA; acetoacetyl-CoA; malonyl-CoA; succinyl-CoA; n-propionyl-CoA; n-butyryl-CoA; n-hexyl-CoA; n-valeryl-CoA.

### Sigmoid Inhibition

An enzyme that displays sigmoidal kinetics will satisfy the Michaelis-Menten equation with the following modification.

\[
\frac{n}{V_{\text{max}}} = \frac{(S)^n}{(K' + (S)^n)}
\]

where \((S)\) is the ligand concentration; \(n\), the interaction coefficient.

Koshland, Nemethy, and Filmer (11) have proposed a little used device that can discriminate between cooperative and noncooperative protein-ligand interaction. They suggested that by determining the cooperativity index \((R_d)\), which is the ratio of substrate concentration necessary to give 90% saturation to that concentration which gives 10% saturation, any deviation from the Michaelis-Menten hyperbolic relationship can easily be determined. \(R_d\) should be 81 for all cases that follow Michaelis kinetics. At \((S)_{90}\) and \((S)_{10}\), Equation 1 can be written in the form of two simultaneous equations as follows.

\[
0.9 = \frac{(S)_{90}^n}{(K' + (S)_{90}^n)}
\]

and

\[
0.1 = \frac{(S)_{10}^n}{(K' + (S)_{10}^n)}
\]

which can readily be reduced to

\[
\frac{(S)_{90}}{(S)_{10}} = 81
\]

From Equation 4, one could determine the nature of interaction...
Fig. 4. Inhibition of the biosynthetic reaction catalyzed by Pythium NAD-specific glutamic dehydrogenase by palmitoyl- and oleyl-CoA and antagonism by the activators, GTP, CoA, P-enolpyruvate (PEP), and NADP⁺ against oleyl-CoA inhibition. Inset, an enlargement of the curves at low oleyl-CoA concentrations. The concentrations of activators used are given in Fig. 2 legend. The expression “All Activators” represents the four activators given above.

between ligand and protein and evaluate the interaction coefficient, n, which is only an approximate indication of the number of substrate-binding sites involved in the interaction.

When a substrate acts as an allosteric inhibitor, theoretically, it should be possible to analyze the saturation curve on the same basis by using Equation 5.

\[
\frac{[S]^*}{[S]^{100}} = 81
\]  

(5)

It must be emphasized that most substrate inhibition curves would give n values greater than 1 when Equation 5 is used. Other diagnostic procedures would be required to confirm whether or not true cooperativity is involved. This approach has some value only when modulators activate an enzyme at high substrate concentration ranges.

The computational procedure we followed was to select as zero inhibitor concentration the concentration of substrate at which there is neither an increase nor a decrease in the reaction rate when the substrate level was increased further by at least 10%. The concentration of substrate required to reduce this optimal rate by 10% was taken to represent \([S]^{10}\) and the concentration of substrate that reduced the optimal rate by 90% taken as \([S]^{90}\).

Analysis of Fig. 3a in which ammonia acted as a substrate and an inhibitor of the reductive amination reaction of Pythium NAD-specific glutamic dehydrogenase, based on these suggestions, showed that the interaction of the inhibitor with the enzyme may be positive cooperative. \([S]^{90}/[S]^{10}\) was estimated as 3 and n value computed as 4. A similar computation done for α-ketoglutarate as an inhibitor and substrate (Fig. 3b) gave an \([S]^{90}/[S]^{10}\) value of 3.72 and n of 3.3.

**Allosteric Activators—NADP⁺, NADPH, and P-enolpyruvate** have been shown to be allosteric activators of *Pythium* and *Achlya* glutamic dehydrogenases (4). We have since discovered several other activators of these enzymes. These are short chain fatty acid coenzyme A esters (CoA, acetyl-CoA, acetoacetyl-CoA, succinyl-CoA, n-butyryl-CoA, n-propionyl-CoA, malonyl-CoA, n-hexyl-CoA, and n-valeryl-CoA), and the guanylates (GTP, GDP, and GMP). ATP, as an activator, has been referred to above under adenylate control. Activation by all effectors was more pronounced on the *Pythium* catalyst than on *Achlya* glutamic dehydrogenase (Table I). The activators were tested for their efficacy in the reductive amination and oxidative deamination reactions of both enzymes. Cyclic nucleotides, 3',5'-AMP and 3',5'-GMP, had slight stimulatory influence on the biosynthetic reaction but inhibited the catabolic process. Besides quantitative differences shown in the influence of the activators on the two enzymes, the effects of adenylates and P-enolpyruvate on these glutamic dehydrogenases were markedly different. *Pythium* glutamic dehydrogenase was inhibited by P-enolpyruvate when the catabolic reaction was catalyzed whereas *Achlya* glutamic dehydrogenase was activated. ATP inhibited the catabolic reaction of *Achlya* glutamic dehydrogenase but not that of *Pythium*. Other than these subtle modifica-
activations, the two enzymes displayed very similar properties although the affinities for the ligands that interact with them differed markedly (4).

Allosteric Inhibitors—In addition to citrate and AMP, long chain fatty acid-CoA esters were found to be inhibitors of these glutamic dehydrogenases (Fig. 4). The two esters studied in some detail were palmitoyl-CoA and oleyl-CoA. (I)½ values for these esters are given in Table II. At very low concentrations, the esters activated the enzyme slightly. This may be caused by the presence of traces of free CoA that are present in the commercial preparation of the esters. Alternatively, the ester may be acting as a competitive inhibitor with one of the substrates that inhibit the enzyme. At higher concentrations, the ester would bind at its own inhibitor site as well.

(SI)½ and Activation—We have mentioned elsewhere (4) that the activation mechanism operative on the glutamic dehydrogenase is geared toward modulation of the enzymatic activity at high substrate concentrations where substrates appear to inhibit allosterically. By studying the influence of effectors on the enzyme at (S)½ of a substrate, a valuable estimate of the extent of ligand activation or inhibition can be made. This approach was taken to evaluate all activators of these glutamic dehydrogenases. The data given here are predominantly for the Pythium catalyst. Ammonia was held fixed at (S)½ and the other substrates kept at optimal levels. c-r-Ketoglutarate, being also an allosteric substrate inhibitor, was not used at saturating concentrations. Saturation curves were determined for each of the following activators: GTP, GDP, GMP, CoA, acetyl-CoA, succinyl-CoA, aceetoacetyl-CoA, malonyl-CoA, n-propionyl-CoA, n-butyryl-CoA, n-hexyl-CoA, n-valerlyl-CoA, and dephospho-CoA. An activation curve for GTP is presented in Fig. 5 and the activation curves for four of the nine short chain acyl-CoA derivatives in Fig. 6. The results are presented as plots of (v - v0) against the concentration of the activators. The notation v0 is the reaction rate with activator and v1 is the rate without activator. The same data were analyzed in Lineweaver-Burk double reciprocal form. This method permitted us to determine the K½ (activation constant) values for the activators and also evaluate the nature of ligand binding. Although the activation of all of the short chain acyl-CoA derivatives were...
Multivalent Regulation of Glutamic Dehydrogenases

Summary

CoA, n-Propionyl-CoA, Succinyl-CoA, Acetyl-CoA, P-enolpyruvate, NADP+, 3',5'-GMP.

Malonyl-CoA, n-Butyryl-CoA, n-Hexyl-CoA, n-Valeryl-CoA.

P-enolpyruvate + NADP+yGTP + CoA

NADP+, 0.167 mM; P-enolpyruvate, 0.5 mM; GTP, 0.33 mM; CoA, 0.167 mM.

66.67 mM Tris-acetate, pH 8, and 4 pg of enzyme in 3 ml volume.

AMP, 0.167 mM; NADH, 6.67 mM; a-ketoglutarate, 20 mM; NH4+, 6.0 X 10^-6 M.

Pythium glutamic dehydrogenases, only NADP+ and P-enolpyruvate could, independently, overcome most of the inhibition (Table IV). GTP, CoA (or acetyl-CoA) did not antagonize AMP inhibition significantly. Combined, GTP and CoA were no better than CoA alone. On the contrary, NADP+ and P-enolpyruvate acted cumulatively and antagonized AMP completely.

Although P-enolpyruvate was cumulative with either GTP or CoA, NADP+ did not show any cumulative property with these two compounds. It would appear from these results that NADP+, P-enolpyruvate and CoA are interacting at the same or closely related sites. P-Enolpyruvate, definitely, has a distinct site. However, because of the small relative differences in percentage deinhibition elicited when GTP and CoA were used, it became necessary to estimate the number of activator sites by using other inhibitors.

Citrate Inhibition—All of the activators were tested for their ability to release the enzyme from citrate inhibition. A previous study had shown that NADP+ and P-enolpyruvate acted cumulatively against citrate (4). Similar studies were done here with GTP, ATP, and acetyl-CoA derivatives and then compared against NADP+ and P-enolpyruvate antagonism.

Although ATP is an activator (based on the adenylate control hypothesis) it failed to antagonize citrate (Fig. 7). GTP, CoA, and P-enolpyruvate were weakly antagonistic to citrate. Only NADP+ showed a significant antagonistic property. GTP and CoA had some cumulative ability, but they were considerably less effective than NADP+ alone. When P-enolpyruvate and

### Table III

| Ligand               | Concentration (mM) | (v/v0) | (4) | a |
|----------------------|-------------------|-------|-----|---|
| NADP+                | 0.0167            | 2.85  | 3.37 x 10^-2 |
| P-enolpyruvate       | 0.50              | 2.0   | 6.0 x 10^-2  |
| GTP                  | 0.333             | 2.70  | 3.3 x 10^-5  |
| Acetyl-CoA           | 0.167             | 2.4   | 3.0 x 10^-5  |
| Malonyl-CoA          | 0.167             | 2.0   | 3.0 x 10^-5  |
| Succinyl-CoA         | 0.167             | 2.0   | 3.0 x 10^-5  |
| n-Butyryl-CoA        | 0.167             | 1.75  | 3.3 x 10^-5  |
| n-Propionyl-CoA      | 0.167             | 1.75  | 3.50 x 10^-5 |
| n-Valeryl-CoA        | 0.167             | 1.75  | 3.50 x 10^-5 |
| n-Hexyl-CoA          | 0.167             | 1.75  | 3.50 x 10^-5 |
| CoA                  | 0.167             | 2.10  | 3.30 x 10^-4  |
| 3',5'-GMP            | 0.333             | 1.15  | 6.67 x 10^-5 |

a Ligand concentration required to fill half of the sites on the enzyme.

### Table IV

| Ligands                  | Inhibition (%) |
|--------------------------|----------------|
| None                     | 100            |
| NADP+                    | 22             |
| P-enolpyruvate           | 12             |
| CoA                      | 68             |
| Acetyl-CoA               | 75             |
| GTP                      | 08             |
| GTP + CoA                | 18             |
| NADP+ + CoA              | 20             |
| NADP+ + GTP              | 0              |
| NADP+ + P-enolpyruvate   | 10             |
| P-enolpyruvate + CoA     | 6              |
| P-enolpyruvate + GTP     | 2              |
| P-enolpyruvate + CoA + GTP| 0              |

analyzed in this manner, the plots given are the curves for CoA, acetyl-CoA, butyryl-CoA, and succinyl-CoA. A curve for diphospho-CoA, which neither activated nor inhibited the enzyme, has been included but the double reciprocal form omitted.

There were relatively little differences in the degree of activation caused by the acetyl-CoA derivatives. The activation constants for GTP and the acetyl-CoA derivatives were approximately the same at about 4 x 10^-5 M. Since acyl-CoA derivatives and free CoA functioned as activators, the sulphydryl group of CoA does not appear to be essential for activation. The 3'-phosphate group of CoA, however, is essential (Table III).

All of the activators displayed what appears to be a linear function in kinetic binding of the activators to the enzyme. But on reploting the data as log (v - v0)/(Vmax - v0) against log of activator concentration, the log plots were biphasic with one slope of n value equaling 1 and a second slope with n values varying between 2 and 4. Only the data for GTP and butyryl-CoA are presented as they represent the extreme cases of the various biphasic log plots obtained. Presumably, some form of cooperative binding of the activators does occur.

Although the activators of these glutamic dehydrogenases are phosphorylated compounds, their structures are so different that a clear picture cannot be obtained from the structures about important functional groups. Phosphate is unlikely to be the single important factor because AMP is an inhibitor and several other phosphorylated compounds tested have no influence on the enzyme. An alternative interpretation is that multiple sites may be involved in the activation process. Tests were done to see if there was any cumulative property of the activators in their interaction with the enzyme.

### Multivalency

The ability of the activators to antagonize the inhibitory action of AMP, citrate, and long chain fatty acid-CoA esters was used as the test model. The reason for this approach rested on an early observation that all of the activators, except ATP, can easily reverse the allosteric inhibition by the substrates, ammonia, and alpha-ketoglutarate. Nonsubstrate inhibitors were more toxic and resisted antagonism by single activators.

AMP Inhibition—When the activators were used singly and in combination to antagonize AMP effects on Pythium glutamic dehydrogenases, only NADP+ and P-enolpyruvate could, independently, overcome most of the inhibition (Table IV). GTP, CoA (or acetyl-CoA) did not antagonize AMP inhibition significantly. Combined, GTP and CoA were no better than CoA alone. On the contrary, NADP+ and P-enolpyruvate acted cumulatively and antagonized AMP completely.

Although P-enolpyruvate was cumulative with either GTP or CoA, NADP+ did not show any cumulative property with these two compounds. It would appear from these results that NADP+, GTP, and CoA were interacting at the same or closely related sites. P-Enolpyruvate, definitely, has a distinct site. However, because of the small relative differences in percentage deinhibition elicited when GTP and CoA were used, it became necessary to estimate the number of activator sites by using other inhibitors.

Citrate Inhibition—All of the activators were tested for their ability to release the enzyme from citrate inhibition. A previous study had shown that NADP+ and P-enolpyruvate acted cumulatively against citrate (4). Similar studies were done here with GTP, ATP, and acetyl-CoA derivatives and then compared against NADP+ and P-enolpyruvate antagonism.

Although ATP is an activator (based on the adenylate control hypothesis) it failed to antagonize citrate (Fig. 7). GTP, CoA, and P-enolpyruvate were weakly antagonistic to citrate. Only NADP+ showed a significant antagonistic property. GTP and CoA had some cumulative ability, but they were considerably less effective than NADP+ alone. When P-enolpyruvate and
either CoA or GTP were used, their cumulative effect did not quite match that of NADP\(^+\) as an antagonist (see Table II for a record of (Z)\(^{0.5}\) of citrate in the presence of various activators). P-Enolpyruvate, GTP, and CoA combined were cumulatively as effective as NADP\(^+\) alone. When all four activators (NADP\(^+\), P-enolpyruvate, GTP, and CoA) were used, complete antagonism of citrate inhibition occurred (Fig. 7). From these results, it appears that all of the activators have separate binding sites.

Oleyl-CoA Inhibition—Long chain fatty acid-CoA derivatives have been studied in detail by Taketa and Pogell (13) with regard to their inhibitory effect on enzymes not related to fatty acid biosynthesis. They concluded that palmitoyl-CoA may act as a detergent during inhibition. Normally, inhibition of this type would lead to an irreversible denaturation of the protein. Zahler, Barden, and Cleland (14) have estimated that the mixed micellar concentration of palmitoyl-CoA is 2 to 4 \(\mu\)M. Dorsey and Porter (15) showed that the critical mixed micellar concentration of palmitoyl-CoA and fatty acid synthetase is 5 \(\mu\)M. Above this concentration, the enzyme was inhibited irreversibly. We do not know what the critical mixed micellar level for Pythium glutamic dehydrogenase and palmitoyl or oleyl-CoA may be. But Pythium glutamic dehydrogenase was markedly inhibited by oleyl- and palmitoyl-CoA at concentrations above 1.5 \(\mu\)M (Fig. 4). Attempts to antagonize oleyl-CoA inhibition by single activators failed. Only NADP\(^+\) had a very slight protective effect. As shown in Table II and Fig. 4 inset, significant protection against oleyl-CoA inhibition occurred when all the activators were present. The enzyme was not inhibited by oleyl-CoA at concentrations below 6 \(\mu\)M. At 10 \(\mu\)M oleyl-CoA concentration, the enzyme was 80% inhibited in the absence of the activators, but only 20% inhibited when they were added.

Significant inhibition always occurred at oleyl-CoA levels above 10 \(\mu\)M whether activators were present or not. It is possible that the coenzyme may inhibit in this range by detergent action. A more critical study of molar ratio of coenzyme and protein that leads to inhibition and how activators alter this ratio would be required to resolve this problem.

**DISCUSSION**

**Physiological Action of Modulators**

At this stage of our studies, it is not possible to give a complete synthesis of the physiological reasons that would account for the multivalent control of glutamic dehydrogenases from these fungi (Oomycetes).

NADP\(^+\) and P-Enolpyruvate—Conclusions drawn from data presented previously (4) on the physiological basis of NADP\(^+\) and P-enolpyruvate activation of Pythium, Achlya, and Saprolegnia glutamic dehydrogenases are not contradicted. The NADP-specific isocitric and NAD-specific glutamic dehydrogenases act cooperatively to maintain a balance of pyridine nucleotides as follows.

\[
\text{Isocitrate} + \text{NH}_3^+ + \text{NADP}^+ + \text{NADH} \rightarrow \text{NAD}^+ + \text{NADPH} + \text{glutamate} + \text{CO}_2 \quad (6)
\]

P-Enolpyruvate may not be involved in this reaction since the substrates can provide sufficient energy to effect the conversion. Under energy-rich conditions when P-enolpyruvate accumulates, GTP and P-enolpyruvate activation of the biosynthetic reaction of glutamic dehydrogenase is a reasonable effect because the citric acid cycle would be operating as a biosynthetic unit supplying its intermediates for amino acids and nucleotides.

**TABLE V**

Some probable relationships between anabolism and modulators of NAD-specific glutamic dehydrogenases of Oomycetes

| Ligands | Biosynthetic and related reactions |
|--------|----------------------------------|
| AMP/ATP | (i) Adenylate control of amphibolic citric acid cycle activity |
|        | (ii) NAD-kinase reaction |
| NADP\(^+\)/NADPH | Transhydrogenase couple of isocitric and glutamic dehydrogenase |
| Short and long chain acyl-CoA derivatives | Fatty acid biosynthesis and end product feedback effects |
| GTP | (i) Fatty acid biosynthesis |
| | (ii) Substrate level phosphorylation |
| | (iii) Gluconeogenesis via P-enolpyruvate |
| P-enolpyruvate | (i) Pyruvate metabolism |
| | (ii) Amino acid biosynthesis |
| Citrate | Citric acid cycle in biosynthesis |
The questions, why do these enzymes display marked sensitivity to inhibition by their substrates, ammonia and α-ketoglutarate; and why do effectors modulate the enzyme only at these toxic levels of substrate, remain unanswered. But two possible explanations can be offered. First, if α-ketoglutarate is liable to accumulate during its production from carbohydrates and transamination reactions of glutamate, then this metabolite may act directly as a substrate and indirectly as an end product (transaminase-glutamic dehydrogenase couple) feedback effector of the glutamic dehydrogenase. One role of the activators would be to relieve the enzyme from allosteric inhibition by substrates and permit continued biosynthesis. The second and more attractive proposal is that substrate control is related to catabolism. The enzyme must have evolved as a catabolic catalyst, because it is subjected to catabolite repression. During deamination reactions, α-ketoglutarate and ammonia may accumulate. This may have led to the development of a product inhibition control mechanism which is observed as substrate inhibition in these studies. The multivalent control by activators may have evolved subsequently to overcome this inhibition when the enzyme has to function in a biosynthetic capacity.

**Acyl-CoA Derivatives**—According to the transhydrogenase hypothesis, NADPH is produced at the expense of NADH. One use to which the NADPH may be put is to synthesize fatty acids. Activation of the enzyme by short chain intermediates of fatty acid synthesis in a manner that favors NADH utilization fits this concept very well. Palmitoyl- and oleoyl-CoA, which may be considered “end products” of fatty acid biosynthesis feedback, inhibit the enzyme. This is a common method of control of the first enzyme involved in most biosynthetic sequences studied. Although citrate is an inhibitor, its effect is qualitatively unidirectional on the catabolic reaction of the enzyme (4). Therefore, the anomaly of citrate inhibition would not contradict acetyl CoA activation of the enzyme. In fact, it does support the concept that the enzyme has catabolic function because citrate can be looked on as an end product.

**GTP**—A reasonable explanation for the unidirectional stimulation of the enzyme by GTP is difficult because many interrelated reactions utilize GTP. For example, substrate level oxidative phosphorylation of succinyl-CoA produces GTP; a GTP linked fatty acid activation enzyme (acyl-CoA synthetase) is present in mitochondria and closely linked to the oxidation of α-ketoglutarate; GTP is utilized during synthesis of P-enolpyruvate from oxaloacetate via P-enolpyruvate carboxykinase action. It is unlikely that GTP has the same function as ATP because the latter compound responds very differently in multivalent studies. The only definite conclusion we can draw is that GTP, like the other activators, activates the enzyme for active biosynthesis.

**Adenylates**—There are two suggestions that satisfactorily account for the effect of adenylates on the enzyme. (a) The adenylate control may reflect the physiological response of the enzyme to the energy state of the cell. Under energy-rich conditions, there would be an ample supply of ATP that could be utilized for biosynthesis. The biosynthetic reaction of the glutamic dehydrogenase, consequently, would be encouraged. (b) ATP may be involved in an NAD-kinase reaction of the type

\[ \text{ATP} + \text{NAD}^+ \rightarrow \text{NADP}^+ + \text{ADP} \]  

which would assure a continued production of NADP⁺ for the transhydrogenase couple of isocitric and glutamic dehydrogenases.

Table V summarizes those physiological reactions that may be directly influenced by the activity of the glutamic dehydrogenase. The list is not exhaustive. We have only presented those interrelations pertinent to the data presented in this communication.

**Enzyo**

Two different mechanisms of allosteric control of NAD-specific glutamic dehydrogenases have been observed among members of the simple fungi, Phaeomyces. (4, 7). A broad sampling covering some 30 different species and all the major orders of this taxonomic group has been made. By a remarkable coincidence, the two complex enzyme control systems are distributed among the fungi in an identical fashion as the only two known pathways of lysine biosynthesis (16). Fungi are unique organisms in that they alone appear to possess these two biosynthetic pathways. A biochemical rationale for this correlation is not yet evident. It is hoped that more work on these and related enzymatic reactions of amino acid biosynthesis in these fungi may throw some light on the evolutionary aspect of our observations.

**Acknowledgments**—We thank Mrs. T. Cleugh for technical assistance in preparing the report.

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Multivalent Regulation of Glutamic Dehydrogenases from Fungi: EFFECTS OF ADENYLATES, GUANYLATES, AND ACYL COENZYME A DERIVATIVES

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J. Biol. Chem. 1970, 245:5569-5576.

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