Metabolic Control and Structure of Glycolytic Enzymes. VII. Destabilization and Inactivation of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Adenosine Phosphates and Chymotrypsin*

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ABSTRACT: Yeast glyceraldehyde 3-phosphate dehydrogenase, in the presence of adenosine triphosphate, experiences (1) instantaneous competitive inhibition and (2) slow dissociation into monomers or dimers. Adenosine triphosphate and certain other adenine-containing compounds have now been shown to markedly increase the susceptibility of glyceraldehyde 3-phosphate dehydrogenase to rapid proteolysis by chymotrypsin.

Native glyceraldehyde 3-phosphate dehydrogenase lost only a little activity after 7 hr at 25°. In the presence of 1 mM adenosine triphosphate or adenosine diphosphate, the loss of activity was much greater; with adenosine 5′-monophosphate it was only moderate. In sharp contrast, in the presence of adenine, or adenosine, or adenosine 3′5′-cyclic monophosphate, the loss was even less than that of the control with no added nucleotide. Chymotrypsin, in the absence of nucleotides, decreased the activity of glyceraldehyde 3-phosphate dehydrogenase to 87% of its original value in 10 min at 25°. However, in the presence of 1 mM concentrations of adenosine 5′-monophosphate, or adenosine diphosphate, or adenosine triphosphate, chymotrypsin decreased the activity of glyceraldehyde 3-phosphate dehydrogenase to only 37, 27, or 10%, respectively, of its original value. Nicotinamide–adenine dinucleotide prevented completely the adenosine triphosphate effect, and adenosine 3′5′-cyclic monophosphate prevented it almost completely. Free terminal phosphate groups, especially terminal pyrophosphate groups, apparently cause the destabilization and increased susceptibility to proteolysis. Two general mechanisms, a feedback inactivation mechanism and a product inactivation mechanism, are proposed for metabolic control of enzyme degradation by the combined action of metabolites and cellular proteolytic enzymes.

A previous report from this laboratory showed that incubation of yeast glyceraldehyde 3-phosphate dehydrogenase with ATP produced *destabilization* which led to dissociation of the enzyme into subunits and *loss of enzymic activity* (Stancel and Deal, 1968). This process has been thoroughly studied and found to be reversible (G. M. Stancel, and W. C. Deal, submitted for publication). Another paper (Yang and Deal, 1969) reported that incubation of yeast glyceralydehyde 3-phosphate dehydrogenase with 3′,5′-cyclic AMP, adenine, adenosine, AMP, ADP, and ATP led to instantaneous *inhibition of the enzymic activity*. A possible role for the 3′,5′-cyclic AMP and ATP *inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase* as mechanisms for control of glycolysis was presented. Further, comparative studies with rabbit muscle glyceraldehyde 3-phosphate dehydrogenase have shown it to be (1) reversibly dissociated by ATP (Constantinides and Deal, 1968a,b; Constantinides, S. M., and Deal, W. C., in preparation), (2) inhibited competitively by 3′,5′-cyclic AMP and other adenine containing compounds (S. T. Yang and W. C. Deal, in preparation), and (3) dissociated into subunits in aqueous solutions at low temperatures (Constantinides and Deal, 1967, 1968a,b).

One question was whether the *destabilization* of yeast glyceraldehyde 3-phosphate dehydrogenase by ATP has *in vivo* significance as a mechanism for control of glycolysis, through either control of enzyme activity or of enzyme level. One argument against the likelihood of the ATP dissociation, as we produced it (Stancel and Deal, 1968) possessing *in vivo* significance in yeast was that low temperatures (0°) were required to produce the maximum effect. Also the process was very slow.

Therefore, without excluding the possibility that control of dissociation by ATP might occur in yeast (perhaps stimulated by the auxiliary destabilizing effect of another substance), we reoriented our approach and looked for significant effects which could be produced by structural destabilizations more subtle than dissociation. One obvious possibility was that ATP might make the enzyme more susceptible to proteolysis, by a much less extensive unfolding than that required for dissociation.

This paper describes experiments in which we have used loss of glyceraldehyde 3-phosphate dehydrogenase activity as a measure of the ability of ATP, and other adenine-containing nucleotide inhibitors (Yang and Deal, 1969), to produce significant structural alterations, thereby enhancing susceptibility to proteolytic degradation. Of a number of proteolytic enzymes tested, chymotrypsin produced the greatest loss of glyceraldehyde 3-phosphate dehydrogenase activity; it was therefore used for all the studies presented here.

We found that ATP quickly and strongly enhanced the susceptibility of yeast glyceraldehyde 3-phosphate dehydrogenase to chymotrypsin action. Based on this and other results in this

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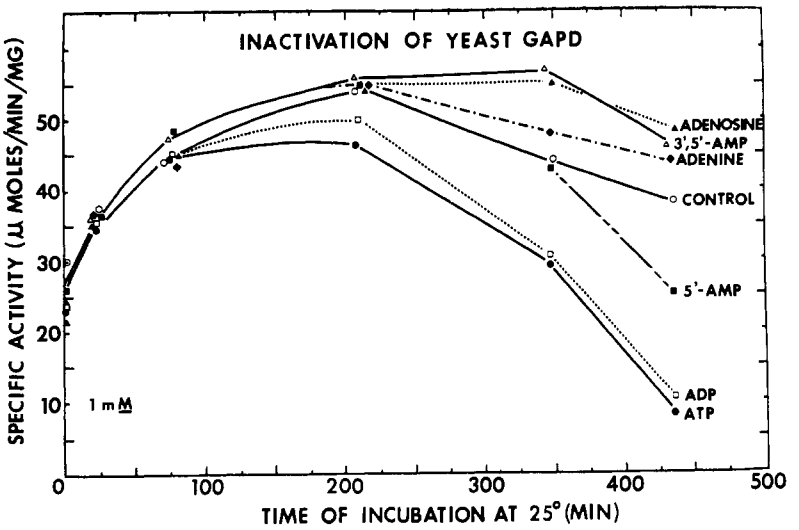

FIGURE 1: Effect of 1 mM adenine, adenosine, and adenosine nucleotides on the spontaneous inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase at 25°. See Methods for details.

paper, we present and discuss two new kinds of mechanisms for control of degradation of enzyme activity: a *feedback inactivation mechanism* and a *product inactivation mechanism.* By means of their control of degradation of enzymes, these two mechanisms provide for possible control of metabolism by the combined action of metabolites and cellular proteolytic enzymes.

Results

All experiments except the first involved incubation of a mixture containing glyceraldehyde 3-phosphate dehydrogenase, chymotrypsin, and the appropriate nucleotide(s) for 10 min. An aliquot from this mixture was then assayed. The resulting dilution essentially stopped the proteolytic degradation and also reduced the nucleotide concentrations to noninhibitory levels (Yang and Deal, 1969). The first tests were controls, to allow comparison of the effects produced by nucleotides alone with those produced by the combined action of nucleotides and chymotrypsin.

Effect of Adenine, Adenosine, AMP, ATP, and 3',5'-Cyclic AMP on the Inactivation of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase. Figure 1 shows the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase as a function of time of incubation at pH 8.5 and 25° with 1 mM concentrations of the series of adenine compounds. During the initial 3–4-hr incubation there was an increase of enzymic activity both with and without these compounds. This activation phenomenon was observed in every solution of yeast glyceraldehyde 3-phosphate dehydrogenase prepared; it was most prominent in samples exposed to room temperature (16–25°).

After about 4-hr incubation the activity of the enzyme control (no inhibitor present) decreased gradually; the activity of the sample containing adenine was slightly higher than that of the control, indicating that adenine slightly stabilized it against the inactivation. In the enzyme samples containing adenosine or 3',5'-cyclic AMP, the enzymic activity started to decrease only after about 6-hr incubation, while those containing ATP or ADP started to decline steeply after 3.5-hr incubation. In the sample containing AMP, the activity also began to decrease after about 3.5-hr incubation, although at a slower rate. Similar results were also observed: (a) at 0°, under otherwise identical conditions and (b) at both 25 and 0°, at a lower glyceraldehyde 3-phosphate dehydrogenase concentration ($^1/_{40}$ of the amount used in the experiments of Figure 1). Thus, AMP, ADP, and ATP were able to gradually inactivate yeast glyceraldehyde 3-phosphate dehydrogenase upon incubation. In contrast, adenine, adenosine, and 3',5'-cyclic AMP protected slightly against inactivation.

Effect of AMP, ADP, and ATP on the Chymotrypsin Inactivation of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase. In the absence of the nucleotides, yeast glyceraldehyde 3-phosphate dehydrogenase was only slightly inactivated by chymotrypsin (Figure 2, control). In sharp contrast, in the presence

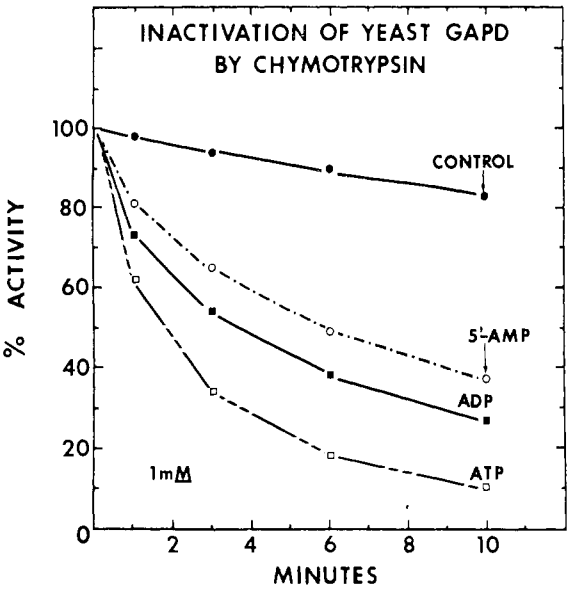

FIGURE 2: Effect of 1 mM AMP, ADP, and ATP on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotryptic digestion. The digestion mixture, containing 50 μg each of yeast glyceraldehyde 3-phosphate dehydrogenase and chymotrypsin and 1 mM of nucleotide (pH 8.5), was incubated at 25°. At intervals of 1, 3, 6, and 10 min, 10-μl aliquots of the digestion mixture were withdrawn and assayed for glyceraldehyde 3-phosphate dehydrogenase activity. See Methods for other details.

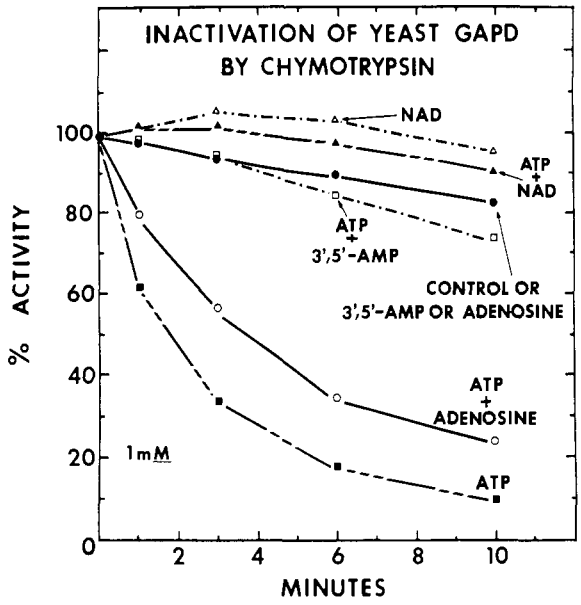

FIGURE 3: Protection of yeast glyceraldehyde 3-phosphate dehydrogenase by 1 mM adenosine or 3',5'-cyclic AMP or NAD against inactivation by ATP and chymotrypsin. See the legend of Figure 1 and Methods for details.

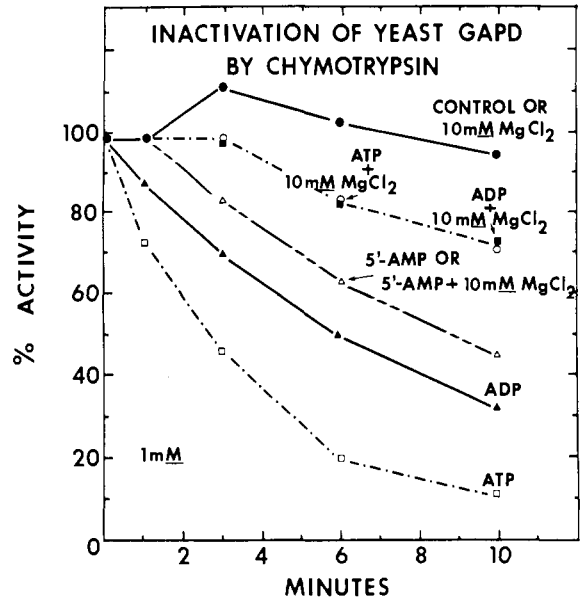

FIGURE 4: Effect of 10 mM of Mg^{2+} ion on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by 1 mM AMP, ADP, and ATP and chymotrypsin. See Methods and text for details.

of 1 mM ATP, glyceraldehyde 3-phosphate dehydrogenase was very quickly inactivated by chymotrypsin; after 10-min digestion, only about 10% of the original glyceraldehyde 3-phosphate dehydrogenase activity was left (Figure 2). In solutions 1 mM in ADP or AMP, yeast glyceraldehyde 3-phosphate dehydrogenase was also rapidly inactivated by chymotrypsin, but at slower rates than with ATP.

The results clearly demonstrated that binding of these nucleotides to yeast glyceraldehyde 3-phosphate dehydrogenase did indeed induce a rapid, although subtle, destabilization or unfolding of certain regions of the enzyme. In times as short as 1 min, the enzyme was destabilized, became susceptible to chymotrypsin digestion, and was inactivated. The order of destabilizing ability (ATP > ADP > AMP) also suggested a correlation between the destabilization effect and the number and/or charge of phosphate groups attached to adenosine. This was further supported by the following experiments.

Stabilization of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Adenosine, 3',5'-Cyclic AMP, and NAD against the Destabilization Effect of ATP. Unlike ATP, and AMP, neither adenosine nor 3',5'-cyclic AMP had any effect on the chymotryptic digestibility of yeast glyceraldehyde 3-phosphate dehydrogenase (Figure 3); the inactivation curves in the presence of 1 mM adenosine or 3',5'-cyclic AMP were identical with that of the control (Figure 3). As expected, NAD completely protected the enzyme against digestion by chymotrypsin in the absence of ATP, ADP, or AMP.

Since the binding affinities of adenosine, 3',5'-cyclic AMP and of NAD were either comparable with or stronger than that of ATP and since they stabilized the enzyme (Figure 1), these compounds were examined for their ability to prevent the enhancement by ATP of the rate of chymotryptic inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase.

As seen in Figure 3, 1 mM NAD completely prevented the enhancement of the chymotryptic inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by 1 mM ATP; 3',5'-cyclic AMP almost completely prevented it. Adenosine also substantially reduced the rate of ATP-enhanced chymotryptic inactivation of the enzyme. The smaller protective effect of adenosine was to be expected since it is not as tightly bound as 3',5'-cyclic AMP or NAD. That is, adenosine has a considerably larger K_i (2.11 mM) than 3',5'-cyclic AMP (0.11 mM) or than the K_m of NAD (0.18 mM) (Yang and Deal, 1969).

Neutralization by Mg^{2+} of the Destabilization Effects of ATP and ADP. As mentioned earlier, the increasing order of susceptibility of yeast glyceraldehyde 3-phosphate dehydrogenase to chymotryptic inactivation in the presence of AMP, ADP, and ATP (Figure 2), together with the lack of effect of adenosine and 3',5'-cyclic AMP (Figure 3), suggested a possible correlation between destabilizing ability and the number and/or charge of the phosphate groups on these nucleotides. (At pH 8.5 the number of negative charges on the monophosphate, diphosphate, and triphosphate esters of the nucleotides, AMP, ADP, and ATP, is expected to be approximately 2, 3, and 4, respectively.)

It also seemed that the specific pyrophosphate group might be required for the greatest destabilization, so an experiment was designed to test this. It has been shown that the phosphate groups of ATP and ADP, but not that of AMP, are able to chelate with Mg^{2+} ion (Bock, 1960; Cohn and Huges, 1962); the pyrophosphate group is apparently required for the complex. Therefore, we examined the ability of Mg^{2+} ion to influence the effects of ATP, ADP, and AMP on the chymotrypsin inactivation of glyceraldehyde 3-phosphate dehydrogenase.

The results are shown in Figure 4. Mg^{2+} ion (10 mM) had no effect on the chymotrypsin digestion of yeast glyceraldehyde 3-phosphate dehydrogenase; the inactivation curve coincided with that of the control. It did not influence the effect of AMP either, which was to be expected from the previous reasoning. In contrast, Mg^{2+} ion dramatically reduced the effects of ATP

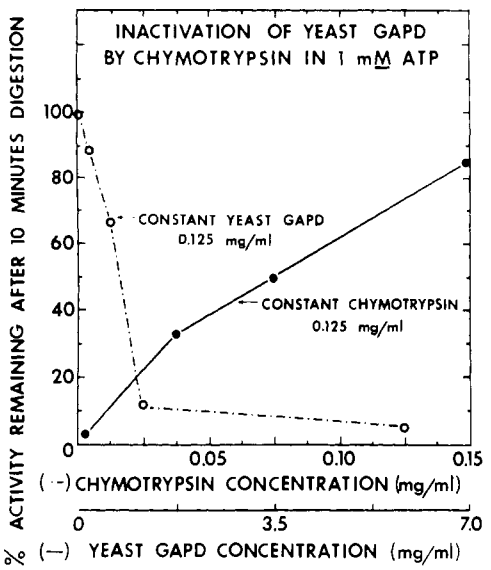

FIGURE 5: Effect of chymotrypsin and glyceraldehyde 3-phosphate dehydrogenase concentrations on the degree of inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by ATP and chymotrypsin in 10 min. The broken curve was obtained by keeping the yeast glyceraldehyde 3-phosphate dehydrogenase concentration constant (0.125 mg/ml) and varying the concentration of chymotrypsin from 0.0042 mg of 0.125 mg/ml. The solid curve was obtained by keeping the chymotrypsin constant (0.125 mg/ml) and varying the concentration of yeast glyceraldehyde 3-phosphate dehydrogenase from 0.125 mg to 7.0 mg per ml. See text for details.

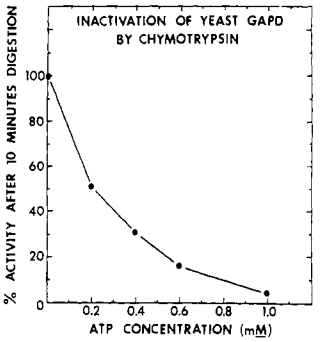

FIGURE 6: Effect of ATP concentration on the degree of chymotryptic inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase in 10 min. The digestion mixture contained 0.125 mg/ml each of yeast glyceraldehyde 3-phosphate dehydrogenase and chymotrypsin and the indicated ATP concentrations. Enzyme activity was assayed after 10-min digestion.

and ADP on the rates of inactivation by chymotrypsin. Furthermore, the curve for ATP plus Mg^{2+} was indentical with that for ADP plus Mg^{2+}. Hence, this suggested that the pyrophosphate groups were very important for the destabilization.

The reduced rates of inactivation could have been due to (1) reduced binding affinities between ATP–Mg^{2+} and ADP–Mg^{2+} complex and the yeast glyceraldehyde 3-phosphate dehydrogenase and/or (2) neutralization of the charges on the phosphate groups of ATP and ADP. Kinetic data (K_i values) showed that Mg^{2+} ion decreased the binding affinity between ATP and yeast glyceraldehyde 3-phosphate dehydrogenase by a factor of one-half. This was not large enough to account for the observed decrease in rate of inactivation. It was, therefore, concluded that the main effect of Mg^{2+} was to neutralize the electrostatic charge of the phosphate groups, rather than to decrease the binding affinity of the nucleotide for glyceraldehyde 3-phosphate dehydrogenase.

Effect of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase: Chymotrypsin Ratio. The effect of variations in glyceraldehyde 3-phosphate dehydrogenase and chymotrypsin concentrations on the rates of glyceraldehyde 3-phosphate dehydrogenase inactivation were analyzed to aid the evaluation of the *in vivo* significance of these effects. Figure 5 shows that with low (0.125 mg/ml) yeast glyceraldehyde 3-phosphate dehydrogenase concentration, the rates of inactivation were high at relatively high chymotrypsin concentrations (0.025 mg/ml and above), but were drastically lower at chymotrypsin concentrations less than this. For the study with constant chymotrypsin concentration (0.125 mg/ml), the rates of inactivation were almost inversely proportional to the glyceraldehyde 3-phosphate dehydrogenase concentration. Significant inactivation occurred at glyceraldehyde 3-phosphate dehydrogenase concentrations as high as 7 mg/ml within only 10 min. This glyceraldehyde 3-phosphate dehydrogenase concentration is probably comparable with the maximum expected *in vivo*.

Effect of ATP Concentration. The effect of ATP concentration on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotrypsin was determined to evaluate further the possible *in vivo* significance of these effects (Figure 6). At 0.2 mM ATP, the enzyme was 50% inactivated after only 10-min digestion. The rate of inactivation was most sensitive to changes in ATP concentration at the lower concentrations.

Effect of Other Compounds. Since the chymotryptic method described in this paper provided a very sensitive test for binding, we retested the compounds previously classed as "nonbinding" on the basis of their lack of inhibition of the enzyme (Yang and Deal, 1969). Figures 7 and 8 show the effects of NADP, deamino-NAD, and NMN on the chymotryptic inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase. At 1 mM concentration none of these compounds exhibited any significant effect on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotrypsin alone (upper

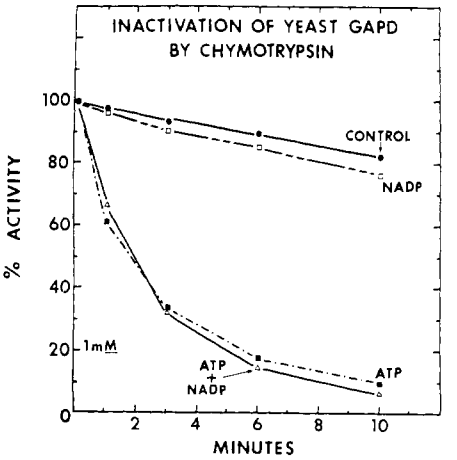

FIGURE 7: Effect of NADP on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by ATP and chymotrypsin. See text for details.

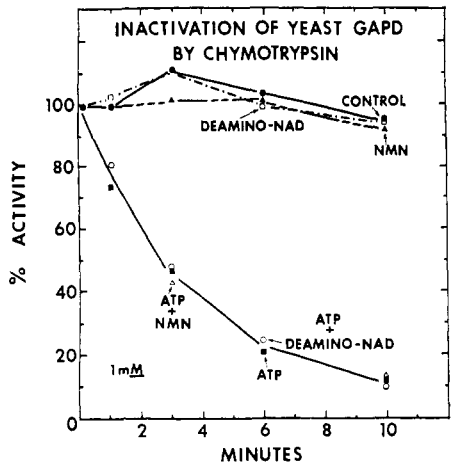

FIGURE 8: Effect of deamino-NAD and NMN on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by ATP and chymotrypsin. See the text for details.

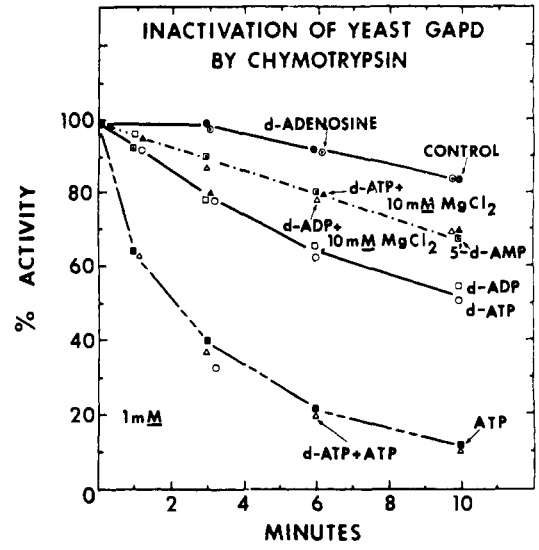

FIGURE 9: Effect of 2′-deoxyadenosine and its nucleotides on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotrypsin, and the effect of Mg^{2+} ion on the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by dADP, dATP, and chymotrypsin. See text for details.

curves, Figures 7 and 8). Thus, these compounds did not appear to bind to yeast glyceraldehyde 3-phosphate dehydrogenase. This conclusion was further supported by the failure of these compounds to affect the inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotrypsin plus ATP (lower curves in Figures 7 and 8).

The nucleoside, 2′-deoxyadenosine, showed no destabilization effect (Figure 9), which was to be expected. However, 2′-deoxynucleotides exhibited substantial destabilization action, as the inactivation curves for dAMP, dADP, and dATP show (Figure 9). These deoxynucleotides seemed to exert their destabilization effect by the same mechanism as that of their nondeoxy analogs (Figure 4), since Mg^{2+} ion also significantly reduced the effect of dADP and dATP (Figure 9). The facts that the 2′-deoxynucleotides produced slower rates of inactivation than the corresponding nondeoxynucleotides, and that dATP had no ability to change the characteristic inactivation curve produced by ATP and chymotrypsin (Figure 9), indicated that the deoxyadenosine nucleotides were only weakly bound to yeast glyceraldehyde 3-phosphate dehydrogenase.

Discussion

The Sequence of Events in Destabilization and Inactivation Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by ATP, ADP, and AMP. The following observations have been made: (1) the rates of *inactivation* of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotrypsin alone or by ATP, ADP, and AMP alone are slow (Figures 1 and 2); (2) the dissociation by ATP of yeast glyceraldehyde 3-phosphate dehydrogenase into dimers or monomers is slow (Stancel and Deal, 1968); (3) the rate of inactivation by chymotrypsin in the presence of ATP is extremely rapid (Figure 2).

These observations suggest the following sequence of events resulting from interaction between yeast glyceraldehyde 3-phosphate dehydrogenase and AMP, ADP, and ATP (also, see Stancel and Deal, 1968): (1) the nucleotides bind to the NAD binding site of the enzyme (Yang and Deal, 1969); (2) this leads to rapid, but slight unfolding of the enzyme in certain specific regions; the initial unfolding is not sufficient to cause disruption of the active site (in the absence of chymotrypsin), or in the quaternary structure of the enzyme, but is sufficient to enhance susceptibility of the enzyme to hydrolysis by chymotrypsin, as shown in this paper; (3) the unfolding process continues and leads to dissociation into subunits which are still fairly compact (Stancel and Deal, 1968); (4) finally, the subunits unfold extensively (Stancel and Deal, 1968).

The Mechanism of Destabilization and Inactivation of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by ATP, ADP, and AMP. The unfolding and inactivation ability of ATP, ADP, and AMP can be attributed to their phosphate groups, or more specifically, to the negative charges of their phosphate groups, especially pyrophosphate groups. This is clearly shown by the ability of Mg^{2+} ion to greatly reduce the effect of ATP and ADP (Figure 4).

The fact that adenosine, 3′,5′-cyclic AMP, and NAD protect the enzyme against the destabilizing effects of ATP, ADP, and AMP supports our previous findings (Yang and Deal, 1969) that all these compounds bind to the NAD binding site on the enzyme and that they do this primarily by interactions involving the adenine nucleotide moieties. The results showing the inability of NADP, deamino-NAD, or NMN to destabilize or stabilize the enzyme also are in good agreement with our findings that the 6-amino group and the 2′-hydroxyl groups of the adenine nucleotide moiety are of major importance in the binding of NAD to the enzyme and that the NMN moiety is not. The previous studies were not able to detect any binding by 2′-deoxyadenosine, dAMP, dADP, and dATP to yeast glyceraldehyde 3-phosphate dehydrogenase (Yang and Deal, 1969). However, the present data (Figure 9) clearly indicate that dATP, dADP, and dAMP do bind (although only weakly), and also that they destabilize yeast glyceraldehyde 3-phosphate dehydrogenase by the same mechanism as AMP, ADP, and ATP. This illustrates the power of the method of "proteolytic inactivation" to detect subtle interactions.

The Significance of the ATP Effect. Our present data (Figure

1) and earlier data (Stancel and Deal, 1968) show that ATP, ADP, and AMP slowly inactivate and dissociate yeast glyceraldehyde 3-phosphate dehydrogenase upon long periods (several hours) of incubation *in vitro* at 0 or 16°. The present experiments were designed to test the possibility that the ATP might cause a more rapid, but perhaps more subtle, conformational change which would greatly increase the rate of proteolytic degradation of yeast glyceraldehyde 3-phosphate dehydrogenase at 25° and thereby control its level.

The degree of inactivation of yeast glyceraldehyde 3-phosphate dehydrogenase by chymotryptic digestion in the presence of these nucleotides was very much greater than in their absence; the activity losses in the presence of AMP, ADP, and ATP were 9, 14, and 19 times, respectively, those of the control after 1 min of digestion (Figure 2). Neither AMP nor ADP would be expected to affect the glyceraldehyde 3-phosphate dehydrogenase inactivation by proteolytic enzymes (or to inhibit glyceraldehyde 3-phosphate dehydrogenase appreciably) because their *in vivo* concentrations are considerably lower than their estimated binding constants. Thus, the *in vivo* concentrations of AMP and ADP are about 0.02–0.16 and 0.1–0.5 mM, respectively, but their K_i values for inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase are 1.1 and 1.5 mM, respectively (Yang and Deal, 1969).

In contrast, the ATP concentration in *most* living systems is probably fairly close to the K_i value of 2.5 mM for the inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by ATP. In yeast cells, the ATP concentration is about 1–2 mM (Betz and Moore, 1967). The *in vivo* concentration of NAD in cells such as liver and yeast is probably near the value of 0.6 mM estimated for rat liver (Jacobson and Kaplan, 1957) and this probably varies with the availability of food supply. (A number of problems associated with measuring free NAD levels (and NAD:NADH ratios) have been discussed by Krebs (1967) and Chance (1967)). Since this NAD concentration is several times the NAD K_m for yeast glyceraldehyde 3-phosphate dehydrogenase and since ATP and NAD compete for the same binding site (Yang and Deal, 1969), the binding by ATP will be small, until the NAD concentration drops considerably below the NAD K_M of 0.18 mM (Yang and Deal, 1969). Such a situation might occur, for example, with overly restricted or overly abundant food supply. Under such conditions, the combined action of ATP and a proteolytic enzyme like chymotrypsin could produce a major irreversible destruction of the enzyme, as this work has shown.

Yeast cells possess several proteolytic enzymes (Felix and Brouillet, 1966; Hyashi *et al.*, 1968), at least one of which is chymotrypsin-like in its catalytic specificity.

The Concepts of Feedback Inactivation and Product Inactivation. We may consider the system composed of the glycolytic enzymes, pyruvate dehydrogenase, the Krebs cycle enzymes, and the electron transport chain to be an ATP-producing system. Since most of the ATP is produced in the terminal steps of this series of reactions, then the effect of ATP on glyceraldehyde 3-phosphate dehydrogenase can be regarded as being of the *feedback* type. By analogy to *feedback inhibition* it is proposed that this type of inactivation mechanism be called *feedback inactivation*, and ATP, which initiates the inactivation reaction, be called the *feedback inactivator*.

Although the feedback inactivation mechanism has not been recognized or emphasized earlier, data suggestive of this type of control mechanism can be found in the literature. For example, Martin (1963) had found that binding of histidine to phosphoribosyl-ATP pyrophosphorylase, the first enzyme in the biosynthetic pathway of histidine, led to destabilization and subsequent inactivation of the enzyme by trypsin. In their studies on the glucose-induced inactivation of malate dehydrogenase in yeast, Duntze *et al.* (1968) attributed the inactivation to a "high-energy metabolite" formed in the *first steps* of glycolysis. This high-energy metabolite might be ATP, and the inactivation mechanism the same as that for yeast glyceraldehyde 3-phosphate dehydrogenase. Malate dehydrogenase, like glyceraldehyde 3-phosphate dehydrogenase, is an NAD-requiring enzyme; thus many of the effects observed with yeast glyceraldehyde 3-phosphate dehydrogenase may also occur with malate dehydrogenase.

Similar reasoning leads to another class of control which we shall designate *product inactivation.* The mechanism is the same as that for *feedback inactivation* except here the initiator is the direct product of the enzyme being inactivated. The product inactivation mechanism can be exemplified by the work of Holzer *et al.* (1967) who found that *Escherichia coli* glutamine synthetase could be inactivated *in vivo* by its reaction product, glutamine, or *in vitro* by glutamine and an inactivating enzyme. Although it has not been fully characterized, we suspect that this inactivating enzyme might be one of the cellular proteolytic enzymes of *Escherichia coli.* Similarly, the inactivation of asparagine synthetase by asparagine reported by Ravel *et al.* (1962) might also be explained by the product inactivation mechanism, rather than by the repression mechanism as suggested by the original authors.

One important aspect of the proposed *feedback inactivation* and the *product inactivation* mechanisms is the postulation of a role for cellular proteolytic enzymes in the long-term regulation of metabolic rates by regulation of enzyme level.

It is probable that some of the enzyme systems now known to show allosteric feedback, or product, inhibition will have their enzyme activity level controlled not only by allosteric inhibition and enzyme repression mechanisms, but also by *feedback inactivation* or *product inactivation. Feedback* and *product inhibitors* which would *repress* enzyme synthesis might also be expected to serve as *feedback inactivators* and *product inactivators* (increasing susceptibility to proteolytic degradation). Hence, a logical, consistent, and integrated control to decrease the capacity of complex metabolic pathways by a key metabolite(s) could be accomplished by: (1) controlling enzyme activity (inhibition), (2) controlling enzyme synthesis (repression), and (3) controlling enzyme degradation (*feedback* or *product inactivation*).

By analogy, converse statements apply to: (1) enzyme activation, (2) enzyme induction, and (3) enzyme stabilization.

In particular, activators or such substances as coenzymes may also increase the rate of synthesis and/or stabilize an enzyme against susceptibility to proteolytic degradation. Such a mechanism has been proposed (Deal, 1967, 1969) for NAD in stimulating the synthesis and preventing the degradation of yeast glyceraldehyde 3-phosphate dehydrogenase. Thus, mechanisms are provided for NAD control of synthesis and degradation of yeast glyceraldehyde 3-phosphate dehydrogenase, with the NAD control of degradation being shared antagonistically with ATP. These postulated mechanisms have the desirable characteristic of versatility in providing simultaneous control of synthesis and degradation of glyceraldehyde 3-phosphate dehydrogenase by NAD.

Materials and Methods

Adenine, adenosine, AMP, ADP, ATP, 3′,5′-cyclic AMP, GTP, and NMN were obtained from the Sigma Biochemical Co. Deoxyadenosine, dAMP, dADP, dATP, NAD, NADP, and deamino-NAD were products of P-L Biochemicals, Inc.

Crystalline yeast glyceraldehyde 3-phosphate dehydrogenase was prepared from Red Star bakers yeast as previously described (Deal, 1969). Solutions of the enzyme at 1-mg/ml concentration were prepared by centrifuging the crystalline enzyme suspension at 17,000 rpm for 20 min, dissolving the pellet in a 0.04 M imidazole buffer (pH 6.9) containing 0.15 M KCl, 8 mM cysteine, and 0.8 mM EDTA, and filtering through fine glass wool. The enzyme solution thus prepared was stable for at least 3 days, when kept at 0°, without appreciable loss of activity or formation of precipitates.

To measure the yeast glyceraldehyde 3-phosphate dehydrogenase activity spectrophotometrically, the previously described procedure (Deal, 1969) was slightly modified as follows: to 280 μl of a 0.05 M glycylglycine buffer (pH 8.5) containing 0.15 M KCl and 0.01 M sodium arsenate, in a microcuvet maintained at 25° were successively added, with mixing, 10 μl of aqueous 0.308 M NAD (pH 6), 50 μl of aqueous 0.038 M cysteine solution (pH 4.4), 50 μl of aqueous 4 mM glyceraldehyde 3-phosphate,[1] and 10 μl of enzyme solution. The total volume of the reaction mixture was 400 μl. It had a pH of 7.8, and the concentrations of NAD, sodium arsenate, glyceraldehyde 3-phosphate, and yeast glyceraldehyde 3-phosphate dehydrogenase were 7.7 mM, 7 mM, 0.5 mM, and 2.2×10^{-5} mM, respectively. The increase in absorbance at 340 mμ, due to formation of NADH, was recorded for 30 sec to 1 min during which a linear reaction rate was always obtained. The units of specific activity used were micromoles per minute per milligram.

The spontaneous inactivation studies of yeast glyceraldehyde 3-phosphate dehydrogenase, as shown in Figure 1, were carried out as follows: to 300 μl of a 0.05 M glycylglycine buffer (pH 8.5) containing 0.15 M KCl and 0.01 M sodium arsenate in a 12 × 75 test tube were added 50 μl of an 8 mM solution of a test compound in the same buffer and 50 μl of yeast glyceraldehyde 3-phosphate dehydrogenase solution. The resulting mixture, containing 1 mM of the test compound and 50 μg of yeast glyceraldehyde 3-phosphate dehydrogenase in a total volume of 400 μl, was incubated at 25°. At intervals, 10-μl aliquots of the mixture were withdrawn and assayed for enzymic activity.

The chymotryptic digestion experiments shown in Figures 2–4 and 7–9 were carried out as follows: to 290 μl of the glycylglycine buffer in a 12 × 75 test tube were added 50 μl of an 8 mM solution of a test compound in the same buffer, 10 μl of a 5-mg/ml, aqueous solution of chymotrypsin (pH 3), and 50 μl of yeast glyceraldehyde 3-phosphate dehydrogenase solution. The resulting mixture, containing 50 μg of yeast glyceraldehyde 3-phosphate dehydrogenase, 50 μg of chymotrypsin, and 1 mM test compound in a total volume of 400 μl, was incubated at 25°. After intervals of 1-, 3-, 6-, and 10-min digestion, 10-μl aliquots of digestion mixture were withdrawn and assayed for enzymic activity. The experiments shown in Figures 5 and 6 were conducted similarly, except the activity was measured only after 10 min.

[1] Ordinarily glyceraldehyde 3-phosphate is always added last (Deal, 1969) since it seems to decompose. But for these studies, it was desirable to add enzyme last to ensure against inactivation before measurement of activity.

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