*RATES OF GDP-INDUCED AND GTP-INDUCED DEPOLYMERIZATION OF GLUTAMATE DEHYDROGENASE: A POSSIBLE FACTOR IN METABOLIC REGULATION**

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Abstract.—The rate of the depolymerization of beef liver glutamate dehydrogenase induced by coenzyme and the purine nucleotides guanosine 5′-diphosphate and guanosine 5′-triphosphate, which are potent inhibitors of enzymatic activity, has been measured by rapid light scattering techniques and by absorbancy changes with stop flow. It is shown that the rate constant for this process may vary from several milliseconds to several seconds depending upon the nucleotides used. The widely varying rate constants for the nucleotide-induced depolymerization may serve a role in determining the nature of the regulation of enzyme activity by nucleotides. Depolymerization induced by guanosine 5′-diphosphate in the presence of diphosphopyridine nucleotide is slower than in the presence of triphosphopyridine nucleotide as coenzyme, and this difference is apparently due to the isomerization of the enzyme as a result of diphosphopyridine nucleotide binding to a second, nonactive site. This binding, as well as binding of the coenzyme to the active site, may be conveniently measured by a purine nucleotide-induced spectral shift in the coenzyme absorption spectrum. It is also shown that complete depolymerization of the enzyme in the presence of guanosine 5′-triphosphate is accomplished by about half saturation of the coenzyme active sites (6–8 active "monomer").

Introduction.—There is increasing evidence that the reversible association-dissociation behavior of some oligomeric enzymes may serve a critical function in the *in vivo* control of catalytic activity and thus be related to the regulation of certain metabolic pathways. There are two issues which are central to the role of the association-dissociation process. One is the question of whether different molecular-weight species display different kinetic parameters, and the other is the question of whether the rate of the polymerization process is fast or slow relative to the rate-determining step in the catalytic reaction. From such considerations it has been postulated that the rate of equilibration between different molecular-weight species may be a factor in determining the type of metabolic control exerted by the enzyme.[1] Polymerization or depolymerization-rate measurements of several enzyme systems in which different molecular-weight species equilibrate slowly have been made, and it is usually observed that such interconversion involves going from an active to a less active or inactive form of the enzyme. Polymerization- and depolymerization-rate measurements for enzyme systems which equilibrate rapidly have not been made previously, and such measurements are the subject of this paper.

Glutamate dehydrogenase serves as a useful model for these studies because it undergoes an easily reversible polymerization reaction. Furthermore, the specific activity is independent of the extent of polymerization, but different molecular-weight species do differ in their ability to bind purine nucleotides. It has been shown previously that this preferential binding of purine nucleotides to different molecular-weight forms of glutamate dehydrogenase gives rise, in binding studies, to an apparent cooperativity with respect to the nucleotides.[2] The importance of this type of preferential binding, assuming rapid equilibration, has been discussed in relation to the regulation and control of enzymatic activity.[3, 4] However, since the rate of equilibration is a critical factor when attempting to correlate binding and kinetic data, it is important to measure such rates.

Methods and Materials.—All experiments were performed at 10°, in 0.1 *M* Tris-acetate buffer, containing 1 mM phosphate and 0.1 mM EDTA. The pH of the buffer was 7.45 at 10°. The enzyme concentration was 1.5 mg/ml (30 μM in terms of active sites) in all experiments.

Molecular-weight changes were observed by mounting a rapid mixer over a Brice-Phoenix light scattering photometer. The dead time of mixing was about 200–300 msec. The changes were followed on an oscilloscope after modifying the photomultiplier circuit to permit these observations and replacing the mercury lamp with a DC operated 100 W Xenon arc. Details of the instrument will be presented elsewhere.

Stop-flow experiments were performed at 310 and 365 mμ using a Durrum stop-flow spectrophotometer with a 2-cm cell. At 365 mμ an additional filter is placed in the mirror box to remove stray light. The absorbancy of DPNH is linear with concentration up to 200 μM under these conditions. Difference spectra were obtained using a Bausch and Lomb Spectronic 600 with two tandem cells and an expanded scale of 80–100% transmittance.

Crystalline glutamate dehydrogenase ($(NH_4)_2SO_4$ suspension) was obtained from Boehringer Corporation. $(NH_4)_2SO_4$ was removed by centrifugation and passing the enzyme over a G-25 column equilibrated with the buffer used for all the experiments described here. Purine nucleotides and coenzymes were obtained from Sigma Chemical Co.

Results.—Active glutamate dehydrogenase has a minimum molecular weight of 300–400,000 and at enzyme concentrations of several mg/ml polymerizes to forms with molecular weights as high as two million or more. Recent studies in fact indicate that the enzyme may polymerize indefinitely.[5–7] Under drastic conditions (urea, acid, etc.), the enzyme may be dissociated into inactive polypeptide chains of 50,000 molecular weight,[8] each chain containing at least one coenzyme binding site as well as one effector site to which purine nucleotides may bind.[3] Kinetic evidence shows that, in addition to the active coenzyme binding site, there may be a second nonactive site for DPNH.[9]

The extent of the polymerization of the active enzyme, aside from being concentration dependent, is greatly influenced by the purine nucleotides in the presence, but not the absence, of coenzyme. In general, those nucleotides which inhibit enzymatic activity bind preferentially to the lower molecular-weight forms, while those which activate bind preferentially to the polymeric forms.[2] Thus, either GTP or GDP, inhibitors of enzymatic activity, essentially prevents the association of the enzyme-DPNH complex. Either DPNH or TPNH alone, at a given enzyme level, increases the extent of polymerization, while GDP or GTP alone has little or no effect on the extent of polymerization. As a result,

the addition of GTP or GDP to the enzyme-coenzyme complex or coenzyme to the enzyme-GDP or GTP complex, at enzyme levels above about 0.2 mg/ml, will "force" the dissociation of the polymer. The major portion of the results presented here deal with the rate of this nucleotide induced dissociation.

Depolymerization induced by DPNH and GDP: Light scattering experiments: Dissociation of glutamate dehydrogenase can occur by at least two distinct processes: (*a*) dissociation by dilution and (*b*) dissociation induced by the binding of nucleotides. The question of dissociation by dilution arises here only because the mixing requires a two-fold dilution of the enzyme on addition of nucleotide.[10]

Figure 1 shows the time dependence of the weight-average molecular weight on addition of varying levels of GDP to the enzyme-DPNH complex. Not only the extent but the apparent rate of dissociation increases as the level of GDP is raised. At the lowest GDP level used (50 μM) the depolymerization process is quite slow, taking close to 50 sec to complete. As the GDP level is raised, this rate becomes faster and at very high levels of GDP (or GTP) it is too rapid to measure by this technique. However, the rapid molecular-weight changes can be measured by absorbancy changes at 310 mμ as described below.

Molecular weight changes as measured by absorbancy changes: (A) DPNH: There are two distinct types of absorbancy changes which may be seen on the addition of purine nucleotides to the enzyme-DPNH complex. One of these represents primarily dissociation and may be observed in the region 300–310 mμ, while the second type of absorbancy change reflects primarily a purine-nucleotide-induced change in the absorption spectrum of the enzyme-DPNH (or TPNH) complex. This latter difference is maximal at about 360–370 mμ. Figure 1 includes (*closed symbols*) the time dependence of the 310 mμ change at varying GDP levels, and it may be seen that there is a good correlation between the 310 mμ change and the light scattering changes. That the absorbancy change in the 300–310 mμ region represents primarily molecular-weight (turbidity) changes while those at 365 mμ are due mainly to a spectral shift is evidenced by the following observations: (1) Dissociation of the enzyme by adding GTP to either the enzyme-DPN or enzyme-DPNH complex produced essentially the same ab-

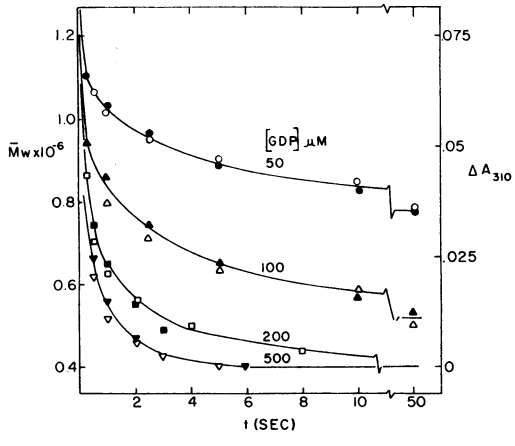

FIG. 1.—Time-dependency of weight average molecular weight (*open symbols*) and absorbancy change at 310 mμ (*closed symbols*) of the enzyme-DPNH complex on addition of varying levels of GDP. Experimental conditions are enzyme, 1.5 mg/ml (30 μM in terms of active sites); DPNH, 150 μM; GDP levels as noted on graph. All experiments were performed at 10° in 0.1 *M* Tris-acetate buffer, containing 1 mM phosphate and 0.1 mM EDTA. The pH of the buffer (at 10°) was 7.45.

sorbancy change at 310 mμ but different changes at 365 mμ; (2) enzyme chemically modified,[2] so as to retain activity and purine nucleotide effects but having lost the ability to polymerize, gives essentially no change in the 310 mμ region on addition of GTP to the enzyme-DPNH complex, although the shift at 365 mμ is still observed; (3) addition of a second mole of DPNH to the dissociated enzyme-GTP-DPNH complex yields no further change at 310 mμ but again reveals the 365 mμ change (see Fig. 2*B*).

Figure 2 shows first order plots of the absorbancy changes at 310 (Fig. 2*A*) or 365 mμ (Fig. 2*B*) on addition of varying amounts of DPNH to enzyme-GTP complex. Since control experiments indicate little or no absorbancy changes on

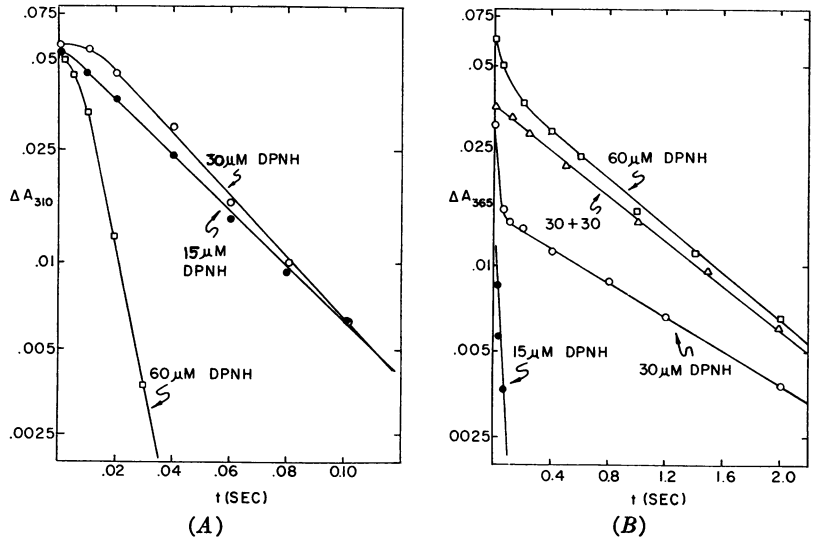

FIG. 2.—First-order plots for absorbancy changes measured by stop flow at 310 mμ (*A*) and 365 mμ (*B*) on addition of DPNH to the enzyme-GTP or enzyme-GTP-DPNH complex. Enzyme concentrations, 1.5 mg/ml (30 μM); GTP, 500 μM. In Fig. 2*A*, added DPNH concentrations are 15 μM (-·-); 30 μM (—○—) and 60 μM (—□—). The same experiments are shown in Fig. 2*B*, except that curve labeled 30 + 30 is the spectral-shift change arising from addition of 30 μM DPNH to a solution already containing enzyme (30 μM), GTP (500 μM), and DPNH (30 μM). For all curves in 2*B*, corrections for turbidity changes have been made except for the 30 + 30 curve in which there is no molecular-weight change occurring on DPNH addition. Buffer as in Fig. 1.

addition of DPNH to the enzyme alone, the results are essentially the same as those that would be obtained on addition of GTP to the enzyme-DPNH complex. Absorbancy changes at 365 mμ which result from changes in the molecular weight of the enzyme are about one half of the magnitude of those observed at 310 mμ. The data obtained at 365 mμ have been corrected for such turbidity changes and therefore represent solely the isomerization process. Coenzyme binding to glutamate dehydrogenase in the presence of GTP is extraordinarily tight, thus making it possible to use the GTP-induced coenzyme spectral shift at 365 mμ to determine the number of coenzyme binding sites. Such titration data,[11] obtained as difference spectra and corrected for turbidity, show that two moles

of DPNH may be bound per mole of polypeptide chain (mol wt = 50,000). Further addition of DPNH to the enzyme-GTP complex produced no further change at 365 mμ. On the other hand, only one mole of TPNH can be bound under the same conditions. These data are consistent with the interpretation of previous kinetic data[9] where it was concluded that inhibition of DPNH oxidation at high DPNH levels was a consequence of DPNH binding to a second, nonactive site. No inhibition of TPNH oxidation was observed at high TPNH levels, and it was concluded that TPNH was unable to bind to this second site. The isomerization of the enzyme which occurs as a consequence of DPNH binding to this second site in the presence of GTP is shown by the line marked 30 + 30 in Figure 2*B*. Here a second equivalent of DPNH (30 μM) is added to an enzyme-GTP-DPNH complex already containing one equivalent of DPNH. The first-order rate constant for the isomerization process under these conditions is 0.77 sec^{-1}. In an experiment such as this, no corrections are needed for turbidity changes since DPNH is added to enzyme which has already been dissociated by the first stoichiometric equivalent of DPNH in the presence of GTP. In fact, examination of Figure 2*A* reveals that dissociation is essentially complete on addition of only about 0.5 stoichiometric equivalents of DPNH to the enzyme-GTP complex. This is consistent with sedimentation studies and holds true for TPNH as well as DPNH.[12] The data imply that the active sites for coenzyme (6–8 per active enzyme monomer) either are not equivalent with respect to the ability to dissociate the enzyme in the presence of GTP or interact in some way. We are not yet able to decide between these two alternatives.

Comparison of the data of Figures 1 and 2*A* reveals that the GDP-induced dissociation of the enzyme-DPNH complex proceeds at a very much slower rate than that induced by GTP. Figure 3 shows a first order plot for the GDP-induced dissociation of the enzyme-DPNH complex. There is a rapid phase followed by a slow dissociation characterized by a rate constant of 0.46 sec^{-1}. The biphasic nature of the plot may reflect different isomerization rates as a con-

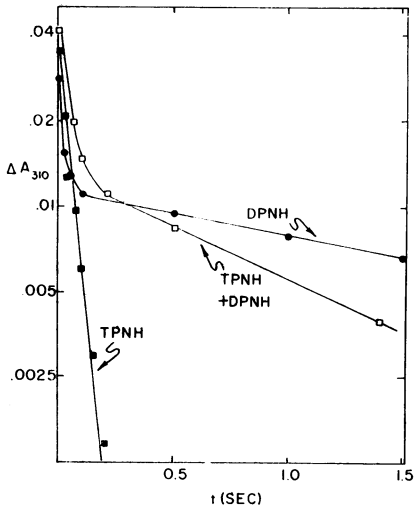

FIG. 3.—First-order plots for the dissociation of the enzyme-TPNH (—■—) or enzyme-DPNH (—·—) complex induced by addition of 200 μM GDP. Enzyme concentration, 30 μM; TPNH, 150 μM; DPNH 60 μM. The third curve (—□—) results from addition of 30 μM DPNH and 200 μM GDP simultaneously to the enzyme-TPNH complex (TPNH = 120 μM). Buffer as in Fig. 1.

sequence of DPNH binding to the first and second binding sites. That the slower rate results from DPNH binding to the second, nonactive site is indicated by using TPNH as coenzyme as described below.

(B) *TPNH:* Figure 3 shows the depolymerization of the enzyme-TPNH complex induced by 200 μM GDP as measured by absorbancy changes at 310 mμ. The first-order rate constant is calculated to be 20 sec^{-1}. The rate constant is relatively constant when GDP is varied from 30 to 500 μM, although the extent of dissociation changes. The time dependence of the change at 365 mμ (not shown here) is essentially identical to the 310 mμ change, but slightly smaller in contrast to the DPNH results (Fig. 2*A*, *B*). The second phase of the GDP-induced dissociation of the enzyme-DPNH complex is about 45 times slower than the TPNH-GDP induced dissociation. We have been unable to find any conditions where the rate constants for the GDP or GTP-induced dissociation of the enzyme-TPNH complex are as slow as observed for DPNH. Since TPNH, unlike DPNH, does not bind to a second site, it would seem possible that the presence or absence of DPNH at this site is the cause for the large differences in the nucleotide-induced depolymerization rates. Figure 3 also shows an experiment where GDP induces the dissociation of enzyme in the presence of both TPNH and DPNH. Here the level of TPNH is four times the enzyme concentration while the DPNH level is equal to the enzyme concentration. It may be seen that the dissociation is considerably slowed by DPNH. Similar results are observed even at lower DPNH levels. It is assumed that the binding constants for TPNH and DPNH to the active site are approximately equal, so that the active site should mostly be occupied by TPNH, while the second nonactive site should be occupied only by DPNH.

Discussion.—Of the number of interesting facts which arise from close examination of the data presented here, most important is the observation that the rate constant for the nucleotide-induced depolymerization of the enzyme-coenzyme complex can vary over a tremendous range. It is also of considerable interest that DPNH binding to a second site can have opposite effects on dissociation rates depending on the nucleotide present. It is apparently responsible for the very slow dissociation induced by GDP and yet dissociation induced by GTP proceeds faster when the second site is occupied with DPNH (Fig. 2*A*). However, it should be pointed out that GDP binds much less tightly to the enzyme-coenzyme complex than does GTP. The levels of GTP used here are saturating while those of GDP are not and at higher GDP levels, the results are similar to those obtained with GTP. The differences observed with GDP and GTP may be related to their relative affinity for the enzyme-coenzyme complex as well as for different molecular-weight forms. In any case, the maximum differences among these rate constants is between the GTP-induced dissociation of the enzyme-TPNH complex ($k_{obs} > 150$ $sec,^{-1}$) and the GDP-induced dissociation of the enzyme-DPNH complex (at low GDP levels, $k_{obs} = 0.14$ sec^{-1}). Thus, the rate constants can differ 1000-fold or more under different conditions. Such differences may have important consequences for the control of enzymatic activity. For example, the initial velocity for a system in which the rate of equilibration between different molecular-weight species is rapid, relative to the

rate-determining step in the catalytic reaction, may show a cooperative type behavior with respect to a ligand which binds differently to different molecular-weight forms.[3, 4] Since the inhibitors, GDP and GTP, in the presence of coenzyme, bind preferentially to low molecular-weight forms of the enzyme,[3] the initial velocity could exhibit a cooperativity with respect to GDP or GTP, which is dependent on the enzyme concentration. Such results have been observed for the GDP inhibition of TPNH oxidation.[2] A system such as this is analogous to the model for allosteric enzymes proposed by Monod *et al.*,[13] which is based on preferential binding of ligands to different enzyme conformations, where an equilibrium exists between different forms. Since the equations derived by Monod *et al.* were for ligand binding, it is always implicitly assumed when applying these equations to kinetic data that equilibration between these different forms is rapid.

However, if such equilibration is slow relative to the rate-determining step in the catalytic reaction, then the conversion of one enzyme form to another (whether these forms are different molecular-weight species or different enzyme conformations) will itself control the velocity of the reaction. The time-dependent inhibition of homoserine dehydrogenase I by threonine recently described by Barber and Bright[14] might represent such a case. Furthermore, the allosteric nature of the response of the initial velocity to ligand concentration may be very different when equilibration rates are slow. It seems quite possible that the slow-rate constants that can be observed for the GDP-induced dissociation of the enzyme-DPNH complex may well be in this range.

Thus, the nature of the response of the enzymatic activity to nucleotide levels may be controlled by the polymerization and depolymerization rates of the enzyme. These rates in turn appear to be controlled by the type and relative concentrations of nucleotides present.

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