COOPERATIVE INTERACTION BETWEEN THE GTP BINDING SITES OF GLUTAMATE DEHYDROGENASE*

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Received November 29, 1965

Guanosine triphosphate (GTP) is known to inhibit the beef liver glutamate dehydrogenase by binding to a specific site distinct from the active site for coenzyme or substrates. Furthermore, evidence from kinetic studies (Frieden, 1963) indicates that GTP is more tightly bound to the enzyme in the presence than in the absence of the coenzymes, DPNH or TPNH. The present study was undertaken in order to measure directly the binding of ^{14}C-labeled GTP with and without added DPNH for comparison with the dissociation constants obtained from kinetic studies. It was observed that in the presence of coenzyme, GTP binds more readily to enzyme molecules which already contain bound GTP; that is, there is cooperative homotropic interaction for GTP (Monod, Wyman and Changeux, 1965). This observation was completely unexpected since such apparent interaction is not detected in kinetic studies using GTP as an inhibitor of the enzymatic reaction (Frieden, 1963). The purpose of this communication is to present data concerning the interaction between GTP sites of glutamate dehydrogenase from the analysis of the binding curves and from sedimentation data at a single high enzyme concentration. Observations concerning the dependence of the interaction on the concentration of the enzyme will also be discussed.

* This investigation was supported by U.S. Public Health Service Research Grant GM 08117-05 and by U.S. Public Health Service Postdoctoral Fellowship 2F2 AM 9576-02 for one of us (R.F.C.).

Experimental - Guanosine 5'-triphosphate-8-^{14}C, 25 mc/mmole , obtained from Schwarz BioResearch, Inc., gave a single radioactive spot on ascending paper chromatography in three solvents. β-DPNH, Grade III was purchased from Sigma Chemical Co. Glutamate dehydrogenase, supplied as a crystalline suspension in ammonium sulfate by Boehringer and Soehne, was subjected to gel filtration on a column of Sephadex G-25, fine grade, to remove ammonium sulfate. The enzyme was equilibrated with 0.1 M Tris acetate buffer, pH 7.15 containing 0.1 mM EDTA and 1 mM sodium phosphate. Under these conditions the enzyme is stable throughout the experiment. GTP-8-^{14}C and DPNH were added as indicated. The protein concentration, in most experiments reported here, was adjusted to 2 mg/ml. Aliquots of these solutions were used to measure the total concentration of GTP in solution. The protein, with bound GTP, was separated ultracentrifugally from the free GTP at 2° by the method of Velick et al. (1953). Aliquots of the supernatants exhibited no enzymatic activity and were dried on metal planchets for determination of radioactivity. A Nuclear Chicago gas flow counter was used and corrections for self-absorption were applied.

The dissociation constants for GTP were determined kinetically as described previously (Frieden, 1963) except that the buffer used was the same as that in the binding studies and the temperature was 16°.

Results and Discussion - The data for the binding of GTP by glutamate dehydrogenase in the absence of DPNH was analyzed graphically in accordance with the equation $r/(GTP)_{free} = n/K - r/K$, where r = moles of GTP bound per 400,000[+] grams, n = number of sites for GTP per protein monomer, and K = dissociation

[+] The molecular weight of the enzymatically active monomer is believed to be 400,000 ± 25,000, somewhat higher than the previously reported value of 350,000 (Frieden, 1965). The present value is derived from light scattering, sedimentation equilibrium and sedimentation and viscosity studies (Colman and Frieden, in preparation; Magar, 1965). Since the enzyme is composed of polypeptide chains with a molecular weight approximately 50,000 (Jirgenson, 1961; Marler and Tanford, 1964), the enzymatically active monomer probably contains eight chains.

constant for the enzyme-GTP complex (Klotz, 1953). This equation, which is valid for the binding of a small molecule by a protein containing multiple equivalent and independent sites, yields a linear plot of $r/(GTP)_{free}$ as a function of r. From the data of Fig. 1A it was determined that glutamate dehydrogenase in the absence of coenzyme contains 8.9 sites per 400,000 grams or approximately one site per peptide chain. The measured dissociation constant is 1.86×10^{-5} M. This compares favorably with the previously predicted kinetic value of $1\text{-}2 \times 10^{-5}$ M (Frieden, 1963).

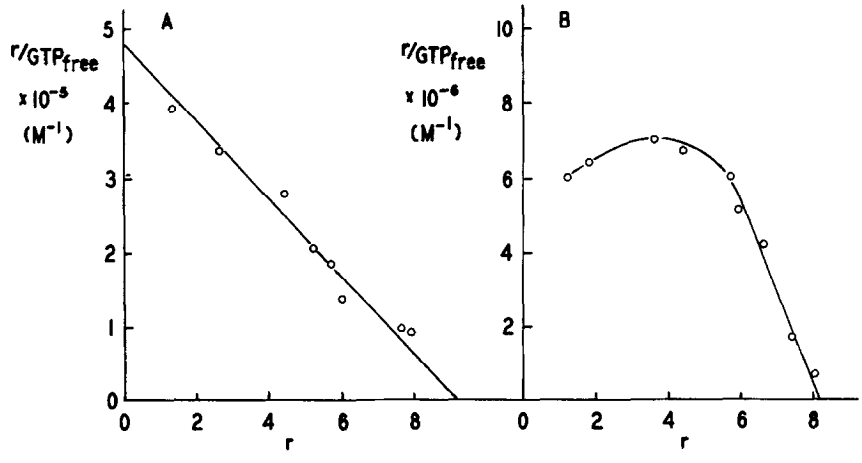

Fig. 1. Plot of $r/(GTP)_{free}$ vs r for glutamate dehydrogenase alone (A) and for enzyme in the presence of $\overline{4} \times 10^{-4}$ M DPNH (B). Experiments were performed at an enzyme concentration of 2 mg/ml. See text for discussion and other experimental conditions.

In the presence of DPNH, the binding of GTP by glutamate dehydrogenase is more complex and the plots of $r/(GTP)_{free}$ versus r are nonlinear, as shown in Fig. 1B. These experiments were conducted with 400 μM DPNH, but similar results were obtained using 100 to 800 μM DPNH. In all cases, the extrapolated value at infinitely high concentrations of GTP ($r/(GTP)_{free} = 0$) gave a value of approximately 8 binding sites for the purine nucleotide, the same as in the absence of DPNH. However, the nonlinearity of the plot indicates that the extent of binding at low concentrations of GTP was con-

siderably less than would be predicted by extrapolating the values at high concentrations of GTP; that is there appeared to be cooperation between GTP sites. It should be noted that a plot of r vs GTP (analogous to a graph of initial reaction velocity vs ligand concentration) would be sigmoidal with an interaction coefficient of 1.6 according to a Hill plot.

Analysis of the data of Fig. 1B shows that at this protein concentration the dissociation constant for the enzyme-DPNH-GTP complex (as obtained from the limiting slope) is 4.1×10^{-7} M, which agrees well with the kinetic value of 3×10^{-7} M derived from GTP inhibition in the presence of DPNH in the same buffer. Added coenzyme thus strengthens the binding of GTP approximately 45-fold as compared with binding by the enzyme alone ($K_{GTP} = 1.86 \times 10^{-5}$ M), a result predicted previously from kinetic studies (Frieden, 1963).

Support for the concept that different forms of the enzyme bind GTP unequally comes from the observation of two discrete peaks in analytical ultracentrifuge experiments involving the enzyme, 100 μM DPNH and non-saturating concentrations of GTP (Magar, 1965; Bayley and Radda, 1965). Table I shows further experiments of this type. It can be seen that there is a correlation between the increase in the average moles of GTP bound per mole of total protein and the increase in the area of the slow peak (13 S) or the corresponding decrease in the area of the fast peak (25 S). This correlation as well as the existence of two peaks suggested that GTP was bound differentially to the two species of the protein.

Using the moving boundary partition cell (Yphantis and Waugh, 1956) in the Spinco Model E Ultracentrifuge, the slow peak was separated from an enzyme preparation to which an average of 4.04 moles of GTP were bound per mole of total protein in the presence of 100 μM DPNH. The measured GTP was corrected for the concentration of free GTP which had been determined in a separate experiment. The slow peak, which represented 46% of the total enzyme contained approximately 6.4 moles of bound GTP per mole of protein or 73% of the total bound GTP. It may be calculated that the fast peak must have con-

TABLE I

Unequal GTP Binding to Different Molecular Weight Species of Glutamate Dehydrogenase

Average Moles GTP Bound/ Moles Total Protein (= r)	Fast Peak	Slow Peak	
	Moles GTP Bound/ Mole Protein	Moles GTP Bound/ Mole Protein	% Total Area*
0.0	0.0	0.0	0%
1.24	-	-	17%
1.96	1.0	5.2	24%
4.04	2.0	6.4	46%
5.72	-	-	78%
7.48	-	-	100%

* The areas given are only approximate (± 5%). They are not corrected for the Johnston-Ogston effect which should be small at this protein concentration (2 mg/ml) but would tend to produce a slow peak which was erroneously large (Schachman, 1959). Since glutamate dehydrogenase undergoes a reversible association reaction, the usual asymmetry of sedimentation was taken into account in calculating the area of the fast peak.

tained a maximum of 2 moles of GTP bound/mole protein. This unequal binding of GTP, which has been confirmed at several concentrations of the purine nucleotide indicates that GTP binds more extensively to enzyme molecules which already contain bound GTP. A certain minimum number of GTP molecules must be bound in order to cause dissociation of the enzyme; otherwise a single peak with a continuously decreasing sedimentation constant would have been observed. It is of interest that in the absence of coenzyme, where there is no evidence of interaction between GTP sites, only one peak is observed in the ultracentrifuge on addition of GTP.

It has been observed that the extent of apparent interaction of GTP binding sites in the presence of coenzyme is dependent on the concentration of the enzyme itself. This observation is currently under investigation, but it is clear that at lower enzyme concentrations, the interaction becomes

less marked. The present data indicate that at the extremely low concentration used in kinetic measurements, the effect disappears or its magnitude falls within experimental error, thereby accounting for the normal (non-sigmoidal) kinetic behavior exhibited when GTP is used as an inhibitor (Frieden, 1963). In addition, further experiments have shown that the extent of apparent interaction is directly related to the well known concentration dependent association-dissociation reaction of the enzyme. Thus the binding properties and undoubtedly the kinetic properties of the enzyme with respect to GTP are affected by the total protein concentration. In this connection it is of importance to note that the concentration of glutamate dehydrogenase in the liver is quite high (>1 mg/gm) and that the kinetic properties of the enzyme under physiological conditions may be very different from those measured in routine kinetic assays. This observation may be relevant with respect to other enzyme systems which undergo reversible association-dissociation reactions and to protein-protein interactions in general.

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