A NEW REACTION OF GLUTAMATE DEHYDROGENASE: THE ENZYME-CATALYZED FORMATION OF TRINITROBENZENE FROM TNBS IN THE PRESENCE OF REDUCED COENZYME*

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Abstract: Glutamate dehydrogenase is shown to catalyze the reaction of trinitrobenzene sulfonate with DPNH or TPNH to yield trinitrobenzene, sulfite, and DPN or TPN. The reaction is stereospecific with respect to removal of deuterium from the reduced coenzyme and is affected by the same purine nucleotides which affect the normal activity. In addition, the reaction is inhibited by α-ketoglutarate. No chemical modification of amino acid residues occurs during the catalysis. It is concluded that the TNBS reaction occurs at the catalytic site of the enzyme.

In the course of an investigation of the modification of glutamate dehydrogenase (GDH) by trinitrobenzene sulfonate (TNBS), it was observed that solutions containing high levels of DPNH and TNBS turned a dark red color over a period of time and that the rate of formation of this color was greatly enhanced by the presence of the enzyme. The purpose of the present communication is to show that glutamate dehydrogenase specifically catalyzes the desulfonation of TNBS using either DPNH or TPNH to produce trinitrobenzene (TNB) and DPN or TPN. As will be shown, the reaction is stereospecific with respect to the removal of hydrogen from the coenzyme and the red color formed is the sulfite adduct of trinitrobenzene.

MATERIALS AND METHODS

Bovine glutamate dehydrogenase, equine alcohol dehydrogenase, and yeast glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Company as crystalline $(NH_4)_2SO_4$ suspensions and were passed over a Sephadex G-25 column

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to remove the ammonium sulfate before use. Glutamate dehydrogenase was assayed as previously described (1). Nucleotides, coenzymes and TNBS were purchased from Sigma and used without further purification. Authentic trinitrobenzene was obtained from K and K Laboratories, Inc. and recrystallized from ethanol twice before use. Deutero-glucose-6-P was a gift from Dr. Luis Glaser. All other chemicals were either analytical grade or spectroscopic grade reagents.

Visible and ultraviolet measurements were made on a Beckman DU monochrometer equipped with a Gilford optical density converter and a recorder having an expanded scale such that full deflection corresponds to 0.1 optical density units.

A Perkin-Elmer 260B was used for infra-red measurements; samples (in $CHCl_3$) were dried over $MgSO_4$, and measured in NaCl cells.

Mass spectra were taken with an LKB 9000 mass spectrometer using the direct probe.

Silica gel G thin layer plates were poured according to Stahl (2) and were developed in one dimension in $CHCl_3$:methanol:water:: 2:3:1. DPN and DPNH were visualized as dark spots and fluorescent spots respectively under ultraviolet illumination; and TNB or the reaction product as a red spot on spraying with a 0.5 M sulfite solution.

TPNH containing deuterium on the B side was prepared by reducing TPN with limiting amounts of deuterated glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase, a B specific enzyme (3). The reaction was allowed to go to completion, inhibited from further reaction by addition of phosphate, and used without further isolation. Isolation of TNB for mass spectroscopy was accomplished by extraction with $CHCl_3$.

RESULTS

Figure 1 shows the time dependence of the disappearance of DPNH in the presence and absence of glutamate dehydrogenase using the conditions stated in the legend. While the reaction does occur non-enzymatically, the rate is considerably enhanced by the presence of the enzyme. Also shown is the effect

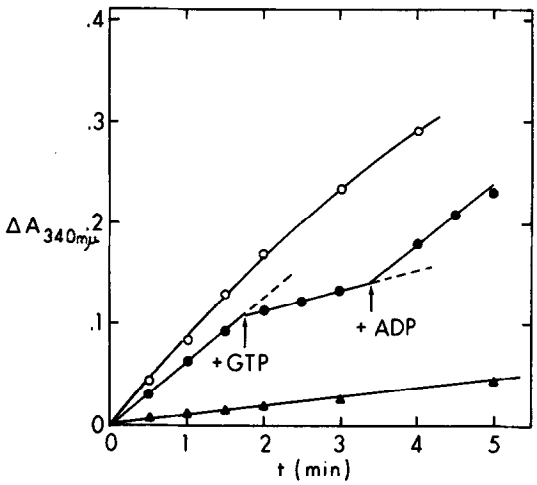


Figure 1. Time dependence of DPNH disappearance (as measured by $\Delta A_{340m\mu}$) in the TNBS reaction in the presence of GDH. Experiments were performed in .05 M phosphate buffer, pH 8, 1.5×10^{-4} M DPNH, 2×10^{-3} M TNBS and 0.04 mg/ml GDH. (-o-), 1×10^{-3} M ADP present initially; (-●-), reaction in absence of purine nucleotides initially and 1×10^{-4} M GTP and 1×10^{-3} M ADP added as indicated by arrows; (-▲-), 1×10^{-3} M αKG added initially. The rate of the reaction in the presence of αKG (lowest curve) is identical to the rate of the non-enzymatic reaction.

of various compounds on the enzymatic rate. GTP, a well-known inhibitor of glutamate dehydrogenase, inhibits the formation of red color, as does α-ketoglutarate. ADP, which actives the normal activity of the enzyme and prevents inhibition by GTP, both activates the TNBS reaction and overcomes the inhibition by GTP. Strong inhibition of the reaction by α-ketoglutarate implies that the TNBS is binding to the same binding site as α-ketoglutarate.

Identification of products

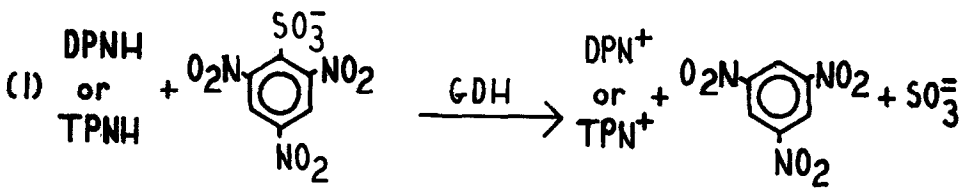
Thin layer chromatography easily separates the two organic products of the reaction mixture. When the plate is sprayed with a sulfite solution, one of the products, which migrates near the solvent front, turns red. The other appears as an absorbing spot under ultraviolet illumination which migrates identically with known standards of DPN.

Further confirmation that DPN was indeed formed in the reaction is obtained by treating the aqueous layer of a chloroform extraction of the reaction mixture with horse liver alcohol dehydrogenase in the presence of ethanol.

At least 80% of the absorbance loss at 340 mμ during the glutamate dehydrogenase reaction was recovered after the alcohol dehydrogenase reaction. The spectrum of this material was identical to DPNH.

It is known that TNB forms a σ complex with $SO_3^=$ which has an absorption band at 462 mμ with a shoulder at 530 mμ, making it red in color (4,5). On the basis of similar absorption bands, we postulated that the other organic product of the enzymatic reaction was trinitrobenzene. Infra-red spectra of pure TNB and the product isolated from the reaction mixture by chloroform extraction were superimposable with absorbance bands at 3110, 1623, 1550, 1345, 1075, and 920 cm^{-1}. Using mass spectrometry, identical patterns were obtained for the reaction product and for pure trinitrobenzene including the formation of a molecular ion at M/e = 213. The mass spectral patterns of the reaction product are the same whether TPNH or DPNH is used as coenzyme for the reaction.

On the basis of these results, we can therefore conclude that glutamate dehydrogenase catalyzes the reaction



and that the red color, which has a maximum at 462 mμ and a shoulder at 530 mμ, arises from the TNB-$SO_3^=$ complex. That the product is sulfite rather than sulfate is shown by the fact that sulfate does not form a red complex with TNB. Addition of an oxidizing agent, such as H_2O_2, to the completed reaction causes disappearance of the 462 mμ and 530 mμ bands. The original spectrum can be regenerated by the addition of $SO_3^=$.

Stereospecificity of the Reaction

In order to determine whether the coenzyme was being utilized the same way in the TNBS reaction as in the normal catalytic reaction, we determined the stereospecificity of the removal of deuterium from deuterated TPNH.

Glutamate dehydrogenase is B specific (6). Table I shows that the deu-

TABLE I

Experiments were performed in .05 M phosphate buffer at pH 8 and 25°, at a TNBS concentration of 1×10^{-3} M and coenzyme concentration of 1×10^{-3} M. The conversion of TNBS to TNB was at least 70% in all cases. The deuterium content of the reaction product TNB was determined from the molecular ion peaks in the mass spectra, corrected for the contribution of ^{13}C. (7)

Coenzyme	Solvent	Enzyme (mg/ml)	Atoms D per molecule TNB
TPNH	H_2O	0.14	0
B TPND	H_2O	1.15	.85
TPNH	D_2O	0.09	.049

terium from the B side of TPNH was directly incorporated into the TNB. That there is not 1 atom of deuterium per molecule of TNB may be due to the fact that there is also a non-enzymatic reaction yielding the same products which, because of isotope discrimination, favors incorporation of H from the A side of the coenzyme. Because there appears to be a large kinetic isotope effect on the enzymatic reaction, the high enzyme concentration was used to minimize the non-enzymatic contribution. Within the accuracy of the instrument, little or no exchange was observed when the reaction was carried out in D_2O, instead of H_2O.

Other experiments indicated that tritium label from the A side of TPNT was not transferred enzymatically to TNB. Also, in spite of the fact that TNBS reacts rapidly with lysine groups of GDH (8,9), essentially no such modification occurs under the conditions used for these experiments.

Of a number of dehydrogenases tested, only the glutamate dehydrogenases appear to catalyze this reaction.

DISCUSSION

The data presented here show unequivocally that glutamate dehydrogenase catalyzes the reaction shown in equation (1). The reaction appears to be catalyzed at the same sites involved in the normal enzymatic reaction since it is effected by the same allosteric effectors as well as inhibited by the substrate α-ketoglutarate. Furthermore, the TNBS at this site does not chemically

modify the reactive lysine groups of the enzyme. The turnover number for the enzyme catalyzed TNBS reaction calculated from the data in Figure 1 is about 100 fold lower than that for the α-ketoglutarate reaction, but this may reflect the subsaturating levels of TNBS used.

This unique reaction will be of great utility in studies of the mechanism of glutamate dehydrogenase because, (1) the reaction now involves two substrates instead of the normal three; (2) rate measurements and isotope rate effects should define more precisely the rate determining step in the overall reaction; and (3) the non-enzymatic reaction, which appears to proceed by a very similar mechanism (unpublished results) should serve as a model for the enzymatic system in terms of proximity and placement of reactive substrate moieties. Studies on these three points are now in progress.

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