TRANSIENT-STATE INTERMEDIATES INVOLVED IN THE HYDRIDE TRANSFER STEP OF THE GLUTAMATE DEHYDROGENASE REACTION*

Harvey F. Fisher, James R. Bard and Russell A. Prough
Veterans Administration Hospital
4801 Linwood Boulevard, Kansas City, Missouri
and the University of Kansas School of Medicine

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SUMMARY

Stopped-flow spectrophotometric studies of the oxidative deamination of L-glutamate by L-glutamate dehydrogenase and TPN show that the rapid first phase or "burst" in absorbance is due to the formation of a previously unreported complex with a maximum absorbance at 332 mμ. The second slower phase consists of the maintenance of a constant level of this blue-shifted complex accompanied by a slow production of complex with a peak at about 348 mμ. Free TPNH release occurs only later as a third stage. Substitution of deuterium for the α-H atom of L-glutamate produces an isotope effect on the first slope of the reaction, but no effect on the burst height or later phases of the reaction. It is concluded that the burst phase represents the hydride transfer step of the reaction and that the 332 mμ peak is probably the resulting enzyme-TPNH-α-ketoglutarate-NH_4^+ complex.

Transient state kinetic studies by Iwatsubo and Panteloni (1) have demonstrated that the glutamate dehydrogenase catalyzed reaction has a biphasic time course. On the basis of observations carried out at a single wavelength, they have attributed the "initial burst" phase of the reaction to the formation of enzyme-TPNH complex and the slower second phase to the release of free TPNH. We report here stopped-flow kinetic studies at varying wavelengths which provide a series of spectra of the reaction mixture at discrete times; these spectra permit the identification of several intermediate complexes and a detailed description of the time course of these complexes in the initial stages of the reaction.

METHODS AND MATERIALS

TPN and Glutamate dehydrogenase were purchased from Sigma Chemical Company. The enzyme was treated with Norit-A as described previously (2) to remove

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tightly bound nucleotide. L-Glutamic acid-$2D_1$ was purchased from Diaprep, Inc. NMR analysis of the α-H position using the assignments of Hochreiter and Schellenberg (3), indicated less than 10% light hydrogen. The contaminating L-glutamic acid-$2H_1$ was removed by stoichiometrically reacting ten percent of the isotopic mixture with TPN and glutamate dehydrogenase. After heat denaturation of the enzyme, the reaction mixture was passed through a layer of Norit-A on a Millipore filter. The pH of the filtrate was adjusted to pH 3.0 and the L-glutamic acid hydrochloride was allowed to crystallize at 0°C. The crystals were rinsed with chilled 50% and 95% ethanol prior to drying. A sample of unsubstituted L-glutamic acid was carried through the same procedure; it showed kinetic behavior in the enzyme system identical to untreated L-glutamic acid.

Stopped-flow measurements were carried out on a Durrum-Gibson stopped-flow spectrophotometer. A Corning #CS57-51 filter was used at wavelengths above 314 mμ to eliminate stray light and fluorescence. The spectrophotometer was interfaced to a Cary Spectro System 100/ on-line 16 bit computer through a high speed analog to digital converter.

All reactions were run in the following manner: one syringe contained 2 mg/ml enzyme in 0.2 M phosphate buffer, pH 7.67; the other syringe contained 800 μM TPN and 100 mM L-glutamate in the same buffer. The temperature was maintained at 25° $\pm$ .05. The slit width was 0.5 mm, the cuvette pathlength was 2 cm and the dead time of the instrument was 4 msec. A spectrum obtained by mixing a known concentration of TPNH with an equal volume of buffer gave extinction coefficients within 2% of true values at all wavelengths employed.

RESULTS AND DISCUSSION

A typical plot of the dependence of the absorbance on time is shown in figure 1. It will be noted that at 320 mμ the reaction consists of three distinct phases, in contrast to the two phases evident in the plot at 340 mμ

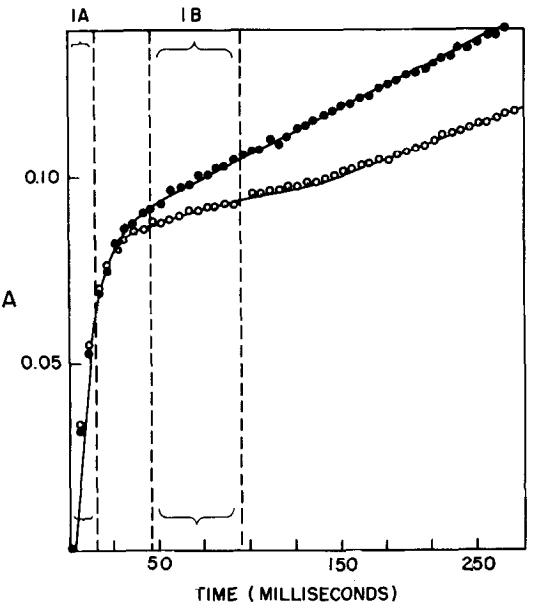

Figure 1. The time dependence of the absorbance of the glutamate dehydrogenase reaction at two different wavelengths. (O) measured at 320 mμ. (●) measured at 340 mμ. Experimental conditions are described in the text.

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as previously reported by Iwatsubo and Panteloni[1]. Spectra synthesized from a series of such curves at times indicated by the vertical lines in Figure 1 are shown in Figure 2. The spectrum shown in Figure 2A, representing the difference in the absorbance of the reaction mixture between 3 msec and 17 msec, is characteristic of the change occurring in the initial "burst" phase of the reaction, and indeed a spectrum obtained from initial rates of time course curves has the precise shape of that of Figure 2A. The spectrum itself resembles the 300-400 mμ band of a blue-shifted TPNH spectrum. While such blue-shifted

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[1] Iwatsubo and Panteloni have attempted to calculate the number of active sites from curves similar to 1B, and have concluded that the enzyme contains 18 to 20 active sites for a molecular weight of 1,100,000. The absorbance value of the "burst" obtained in our experimenta at 340 mμ is somewhat lower than they obtained, but when the TPNH concentration is calculated on the basis of a 2 cm pathlength (the same as used by Iwatsubo and Panteloni) we find only about 6 to 8 micromoles of TPNH produced in the burst, or about 2 per β unit. However, since we will show here that the product formed does not have the same spectrum as that of TPNH, there is no reason to believe that the extinction coefficient of TPNH is applicable.

We acknowledge the assistance of Mr. Joseph Simone in programming the computer.

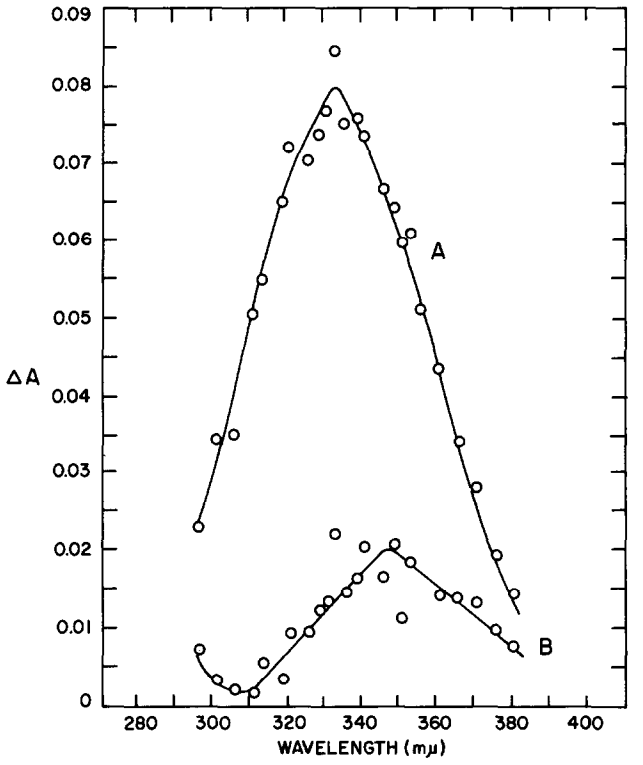

Figure 2. Time-difference spectrum of the reaction. A. 3 to 17 msec. B. 47 to 97 msec. These time periods are indicated in Figure 1 by the appropriately labeled bracketed lines.

spectra have been reported for the open conformation of TPNH (4) and for certain stable complexes between TPNH and A stereospecific dehydrogenases (5), we have not previously observed such a blue shift among the various complexes formed between glutamate dehydrogenase, TPNH and other ligands (2).

Iwatsubo and Panteloni have ascribed this first phase absorbance to the glutamate dehydrogenase-TPNH (ER) binary complex. Figure 2A shows that this initial step cannot be due to an *ER* complex, since such a complex is characterized by a red shift (6). Conceivably the spectrum could be due to a charge transfer complex between enzyme and TPN (*EO*) as has been reported for glyceraldehyde 3-phosphate dehydrogenase (7). Such a charge transfer complex may, however, be ruled out as a cause of the spectrum of Figure 1A, since the spectrum of the *EO* complex of glutamate dehydrogenase contains no such feature in the 300 mμ

region. A charge transfer complex of the composition enzyme-TPN-glutamate (*EOG*) and any possible isomeric forms of such a complex (*EOG'*) are not, however, excluded by data cited thus far. The species responsible for the spectrum of Figure 1, and thus for the burst phenomenon, may be any or all of those included under the brace in Figure 3. The results of the experiment described below, however, will permit us to distinguish between these possibilities.

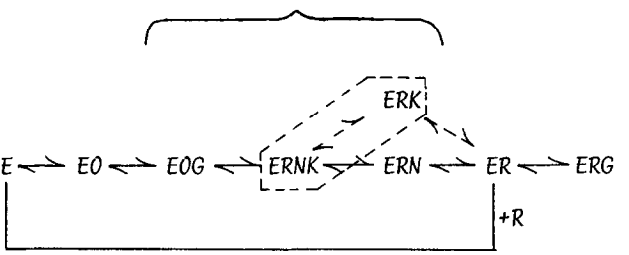

Figure 3. Enzyme-coenzyme-reactant complexes of the glutamate dehydrogeanse reaction. (*E*) enzyme; (*O*) TPN; (*R*) TPNH; (*G*) L-glutamate, (*N*) NH_4^+; (*K*) α-ketoglutarate.

As shown in Figure 4, deuterium substitution of the α-hydrogen atom of L-glutamate causes a significant decrease in the slope of the burst phase but little change in either the eventual heights of that burst or in the slope of the second phase. The first phase isotope effect is 1.5 to 1.8. The existence of such an effect leads to the conclusion that a carbon-deuterium bond must be broken at some step prior to the formation of the species which gives rise to the spectrum of Figure 2A. This species then must occur after complex *EOG* in the scheme of Figure 3. The remaining possible species include only the enzyme-TPNH-NH_4^+ complex (*ERN*) and the enzyme-TPNH-NH_4^+-α-ketoglutarate complex (*ERNK*), and possibly the *ERK* complex proposed recently by Engel and Dalziel (8),as well as possible isomeric forms of such species. However, since we have found that NH_4^+ has no effect on the spectrum of *ER* it is very unlikely that the 320 mμ peak contains significant contributions from *ERN*.

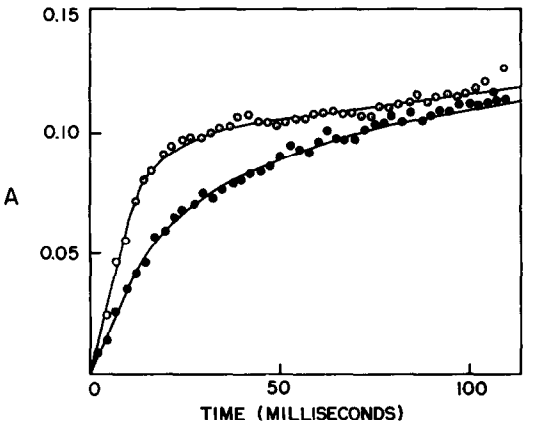

Figure 4. The effect of deuterium substitution of the α-H atom of L-glutamate on the reaction. (○) unsubstituted L-glutamate. (●) L-glutamate-$2D_1$.

We have thus limited the region of likely complexes to the region of the scheme enclosed by the dotted line in Figure 3; only *ERNK* itself and the hypothetical *ERK* remain. It is, therefore, most probable that in the "burst" phase of the reaction we are observing the hydride transfer step itself.

Figure 2B is the time-difference spectrum of the reaction between 47 and 97 msec, the early portion of the "second phase" of the reaction as described by Iwatsubo and Panteloni. It is essentially a red-shifted TPNH 340 mμ band. It may contain spectral contributions from *ER*, *ERG* and *ERN*, all of which have been shown to possess just such red shifted spectra. It is certainly not free R as suggested by Iwatsubo and Panteloni. Production of free TPNH begins only after 150 msec. This second phase cannot be explained as a simple conversion of blue-absorbing species to a red-absorbing one, since the resulting blue-to-red shift would produce a difference spectrum containing a negative component with a minimum at 332 mμ. No such feature is observed. Therefore, if a blue component is converted to a red one, more blue complex must be formed. It is difficult to account for the observed phenomena without assuming separate sites for the two complexes.

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