AN INTERMEDIATE IN THE pH-INDUCED DISSOCIATION OF GLUTAMATE DEHYDROGENASE

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

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We have carried out light-scatter and spectrophotometric stopped-flow studies on the acid induced dissociation of bovine liver L-glutamate dehydrogenase. The light-scatter intensity and the absorbance due to chromophore exposure follow the same time dependence, both changing only after a time lag of about 40 msec. The kinetics are consistent with a simple consecutive, two-step, irreversible reaction in which the first step converts β subunits into other forms identical in molecular weight and degree of folding and is followed by a second step in which these altered forms are converted into random coil, fully dissociated peptide chains.

Several years ago we presented evidence that β subunits of bovine liver L-glutamate dehydrogenase dissociate into δ_2 subunits (unfolded peptide chains) at pH's below 3 or above 9, and that the breaking of one or more tyrosyl, side-chain carboxyl hydrogen bonds was necessary and sufficient to induce this process (Fisher, McGregor, and Cross, 1962). We show here that the step (or steps) involving both the dissociation into subunits and the unfolding of those subunits are preceded by an obligatory pH dependent step which involves neither a change in molecular weight nor a gross exposure of buried aromatic residues.

METHODS

Preparation of the enzyme, protein concentration determination, stopped-flow apparatus and procedures, pH and temperature control, have been described previously (Fisher and Bard, 1969). Light-scatter intensity was measured using the same cuvette in the fluorescence mode. Instrument dead time was determined by the method of Hammes and Haslam, 1968, and was found to be 4 msec. The cuvette was thermostatted at $8.0 \pm 0.1°C$. The monochromater was calibrated using a mercury lamp. Absorbance measurements were made with 0.2 mm slit width (1 mμ band pass), a time constant of 1.0 msec, and oscilloscope

settings of 50 mV/division vertically and 20 msec/division horizontally. Fluorescence measurements were made using a slit width of 4.6 mm (13.8 mμ band pass), vertical setting of 100 mV/division. In each experiment one drive syringe contained the indicated concentration of enzyme in 0.01 M phosphate buffer, pH 7.6; the other drive syringe contained 0.06 M phosphoric acid, pH 1.8. After mixing equal volumes of these two solutions, the final pH was 2.0. Each point in Figure 1 is an average of data from ten experiments. The kinetic constants in Table I were evaluated using the Non-Linear Least Squares program from the BMDX85 Biomedical package developed by the University of California at Los Angeles, using a General Electric 635 computer.

RESULTS AND DISCUSSION

The essential phenomena are demonstrated in Figure 1, in which the time course of changes in both absorbance and light scatter of the solution are followed after rapid mixing.

The total change in absorbance at 289.4 mμ (closed circles) corresponds closely to the absorbance difference of the same concentration of enzyme measured at pH 7.6 vs. pH 2.0. Thus, we are assured that the time dependent absorbance change shown in Figure 1 includes the complete absorbance change and thus represents the exposure of buried tyrosine and tryptophan residues caused by the complete unfolding of a mixture of α and β forms of the enzyme. The change in light scatter at this wavelength is quite small in comparison with the chromophore changes. Similar measurements at other wavelengths show that the signal does indeed represent a pH difference spectrum of glutamate dehydrogenase.

The open circles in Figure 1 represent measurements on the same system, but with the instrument operating in the fluorescence mode with an excitation wavelength of 246.4 mμ, a point at which the ratio of scatter intensity to fluorescence excitation is at a maximum. We have previously demonstrated that α and β glutamate dehydrogenase undergoes a dissociation to δ subunits at low pH, and while we cannot equate scatter intensity directly to static measurements as we did above for the absorbance changes, the ratio of the scatter signal for the pH change to that of the dilution split at constant neutral pH is about the same as the ratio of scatter intensities obtained for the same process on a calibrated light scatter photometer. The open circles, then, represent the time course of the change in weight-average molecular weight

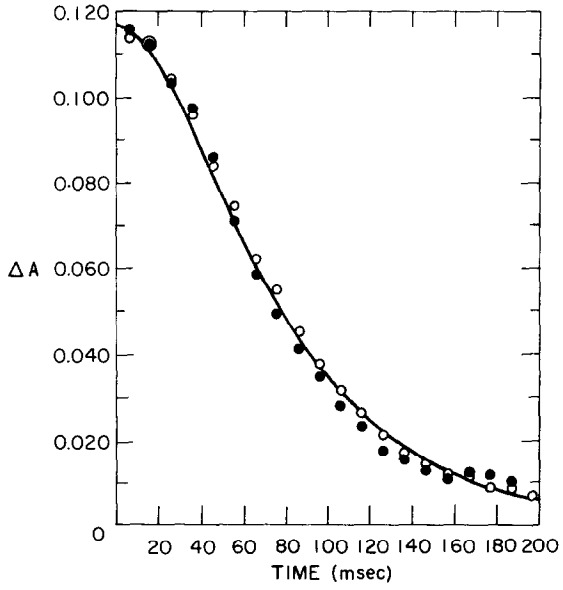

Figure 1. The time dependence of the dissociation reaction. (●) and left hand ordinate — differential absorbance at 289.4 mμ. (○) and right hand ordinate — light scatter intensity at 246 mμ in arbitrary units. Zero absorbance is defined as the absorbance of the enzyme solution at pH 2, $t = \infty$ (trigger shot). Zero scatter intensity is defined as the scatter intensity of the enzyme solution at pH 2.0, $t = 0$. The enzyme concentration was 0.50 mg/ml before mixing, 0.25 mg/ml after mixing.

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caused by rapid lowering of the pH. Again, the change in weight-average molecular weight caused by the 2:1 dilution itself is very small compared to the entire signal resulting from the pH change.

The most obvious features of Figure 1 are: 1. the light scatter changes and the absorbance changes appear to have the same time dependence, and 2. there is a very definite lag in the onset of these two signal changes.

The solid line in Figure 1 represents the following equation:

$$\Delta A = A - \frac{A}{k_2 - k_1}\left[(k_2 - k_2 e^{-k_1 t}) - (k_1 - k_1 e^{-k_2 t})\right] \qquad (1)$$

where t = time in milliseconds, $k_2 = 0.02 \pm .005$ $msec^{-1}$, $k_1 = 0.03 \pm .005$ $msec^{-1}$, A is the total signal change from $t = 0$ to $t = \infty$, and ΔA is the signal change at t minus that at $t = 0$. Equation (1) represents the reaction

$$A \xrightarrow{k_1} B \xrightarrow{k_2} nC \qquad (2)$$

for the case in which only the conversion of B to C produces a signal. The close fit of both sets of data to equation (1) using a single pair of constants

is satisfied by a reaction:

$$\beta_1 \xrightarrow{k_1} \beta_2 \xrightarrow{k_2} n\delta_2 \qquad (3)$$

in which the first step is a process not involving either dissociation into subunits or gross unfolding; while the second step produces fully dissociated, completely unfolded peptide chains. It must be noted that equation (1) is completely symmetrical in k_1 and k_2. While both constants can be evaluated from the data in Figure 1, the assignment of one or the other as representing the first or second step in equations (2) or (3) is completely arbitrary. The direct dissociation of β_2 forms into completely dissociated and unfolded δ_2 subunits in the second step of the reaction does not, of course, preclude the existence of δ_1 (dissociated, but still folded peptide chains) as intermediates in the reaction, but does indicate that if any such intermediates are formed, they are converted to δ_2 subunits so rapidly that they do not affect the observed kinetics.

The dependence of the rate constants on total initial protein concentration is shown in Table I. Constants calculated from absorbance measurements agree with the corresponding constants calculated from light scatter measurements; in each case k_1 is equal to k_2; and, within experimental error, both constants

TABLE I

Dependence of Kinetic Constants on Protein Concentration*

Initial Protein Concentration (mg/ml)	Measurement	k_1 $msec^{-1}$	k_2 $msec^{-1}$
0.26	Absorbance	0.024	0.024
	Scatter	0.024	0.024
0.50	Absorbance	0.024	0.024
	Scatter	0.023	0.023
1.0	Absorbance	0.021	0.021

*Experimental conditions are described in the text.

are practically independent of protein concentration as required by equation (2). Presumably, the irreversibility seen here is only apparent, and the reaction is driven in the direction of dissociation by the high H^+ concentration. Indeed, preliminary observation at somewhat higher pH's indicate such reversibility as well as a hydrogen ion concentration dependence of one or both rate constants. Nevertheless, the driving conditions employed here, by simplifying the mechanism, permit us to demonstrate the existence of an intermediate without ambiguity.

There is one other point to be derived from the lack of a significant concentration dependence. The range of concentrations covered in Table I is one in which the ratio of α to β forms changes rapidly. If β subunits were able to undergo the process described in equation (3) at a rate significantly different from that of the α form of the enzyme, that fact would be evidenced by a large concentration dependence. Its absence suggests that either α or β units dissociate independently and without any obligatory interconversion, or that the α,β interconversion is complete at this pH in about a millisecond. Recent studies from this laboratory on the kinetics of the α,β dissociation make this latter possibility seem less likely (Fisher and Bard, 1969).

Our earlier conclusion that the breaking of tyrosyl-side chain carboxyl hydrogen bonds is necessary and sufficient for the $\beta \longrightarrow \delta_2$ reaction was based on the pH dependence and coincidence of static measurements of the same two signals that we show here occur only after a measurable time lag. That conclusion as stated in those terms remains valid; the results presented here show that, prior to dissociation and unfolding, β units must undergo some fairly slow process which converts them into subunits which are unaltered in molecular weight and degree of folding, but which now immediately undergo a dissociation and unfolding reaction of which the original β units were incapable. We designate these altered subunits as β_2 subunits. The nature of that initial step, represented by k_1, is now under investigation.

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