STUDIES ON HIGH MOLECULAR WEIGHT MODIFIERS OF THE NONHYPERBOLIC *V* VERSUS [*S*] CURVES OF DT-DIAPHORASE

P.M. HOLLANDER and S. GATT

Dept. of Biochemistry, Hebrew University, Hadassa Medical School, P.O.B. 1172, Jerusalem, Israel

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1. Introduction

DT-diaphorase (EC 1.6.99.2) from rat liver catalyzes a bisubstrate reaction in which NAD(P)H is oxidized by various one and two electron acceptors such as potassium ferricyanide or 2,6-dichlorophenolindophenol (DCPIP) [1]. Previous studies have shown that the *V vs* [S] curves of this enzyme are not hyperbolic: The *V vs* [NADH] curve has an intermediary plateau region at about 0.3 mM substrate [2,3] and the *V vs* [DCPIP] plot has a 'trough' (inverted peak) region between approx. 25–37 μM of this acceptor [4]. The complex kinetic curves have been interpreted as reflecting slow isomerization between two enzyme species each having two sites or possibly negative and positive cooperativity between subunits or sites of a multisite enzyme [4]. Temperature, pH, salts [4], ethanol and sucrose Hollander, unpublished results) affected both the *V* vs [NADH] and the *V* vs [DCPIP] curves. Previous experiments showed that nonionic detergents (Tween 20) [1] and phospholipids [5] increased the enzyme dependent reaction rates. These substances also abolish the intermediary plateau of the *V* vs [NADH] plot [2,3,5]. The present report further describes the effects of these compounds as well as of some other high molecular weight substances; such as bovine serum albumine (BSA) and glycogen on the *V* vs [S] curves.

2. Materials and methods

DT-diaphorase was prepared from the soluble fraction of rat liver homogenates, essentially by the method previously described by Ernster et al. [1] and modified as described [4]. The assay medium, in a volume of 1 ml consisted of 50 mM Tris–chloride pH 7.5, NADP and DCPIP and modifier. 5 μl either pretreated or native enzyme was added and the reaction was followed at 37°C, by measuring the decrease in concentration of DCPIP at 600 nm in a Zeiss PMQ II spectrophotometer. The rate of the non-enzymatic reduction of DCPIP by NADH was measured and deducted from the final reaction velocity. Concentrations of NADH solutions were checked at 340 nm and of DCPIP at 600 nm using extinction coefficients of 6.22 and 21 mM^{-1} cm^{-1} respectively. In an alternate procedure the enzyme was pretreated with modifiers as follows: The enzyme was diluted 1:1 with the modifier to give the desired effector concentration. The mixture was maintained at 0°C for 30 min in the dark and 5 μl were added to an assay mixture of buffer, NADH and DCPIP. The modifier was thus diluted 200-fold. Controls to these experiments were obtained with enzyme which was similarly diluted with buffer and kept for 30 min at 0°C in the dark.

3. Results

3.1. *The effect of several members of Triton X-serie on the V vs [S] curves.*

Triton X-67, Triton X-305 and Triton X-405 (0–1.5 mM) were added to the reaction mixtures. None of these substances changed the shapes of the kinetic curves or substantially affected the reaction rates. In contrast Triton X-100, affected both the *V* vs [NADH] and the *V* vs [DCPIP] plots (fig.1), at concentrations above 0.3 mM when the detergent was added to the test system.

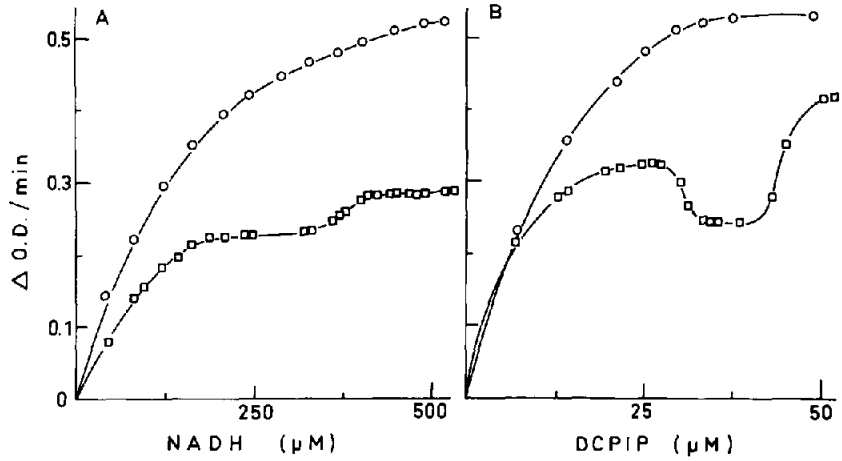

Fig.1. Effects of 1 mM Triton X-100 on the V vs $[S]$ curves. (□–□) control, (○–○) 1 mM Triton X-100 added to the assay system. (A) NADH as varying and 30 μM DCPIP as fixed substrate. (B) DCPIP as varying and 800 μM NADH as fixed substrate.

In separate experiments the enzyme was pretreated with Triton X-100 (1 mM) as described in the Materials and Methods. This again led to V vs [S] curves similar to those in fig.1. Studies of the effect of the Triton X-100 concentration (fig.2) on the reaction rates showed that the greatest increase in the stimulation as well as the most pronounced change in the V vs [S] curves occurred at 0.2–0.3 mM of the detergent, i.e. at its micellizing concentration (6). The effect of another 'soluble' amphiphilic lipid, lysolecithin again showed a maximal effect at its critical micellar concentration (20–40 μM, fig.2) [7]. The effects of Triton X-100 on the enzyme could be reversed by dialyzing out the detergent from the pretreatment mixture.

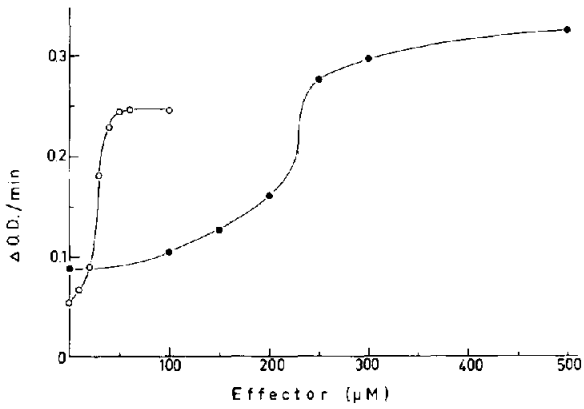

Fig.2. Effects of the concentration of soluble amphiphiles on the initial reaction velocity. (○–○) lysolecithin, (●–●) Triton X-100. Experimental conditions: 1 mM NADH and 32.5 μM DCPIP.

3.2. *The effect of BSA on the initial rate vs [S] curves*

Addition of 9–12 μg/ml BSA into the test system which contained 3 μg protein, transformed the V vs [S] curves into hyperbolas. Lower concentration of BSA increased the reaction rates without affecting the shapes of the kinetic curves.

Pretreatment of the enzyme with BSA gave the same abolishment of the intermediary plateau and trough regions (fig.3A and 3B) as the direct addition of the substance to the test system. Although BSA adsorbs DCPIP, the experiment with preincubation of the enzyme with BSA proves that the changes in the curves are not due to adsorption of one of the substrates but rather to an interaction with the enzyme.

3.3. *Effect of glycogen and lecithin on the kinetics*

Addition of either 1 mg/ml glycogen (mol. wt 10^6–10^7) or 1.0 mM lecithin, dispersed as small liposomes (mol. wt 10^6–10^7) [8] changed the V vs [S] curves into apparent hyperbolas. Pretreatment with these substances had similar effect (fig. 4A and 4B).

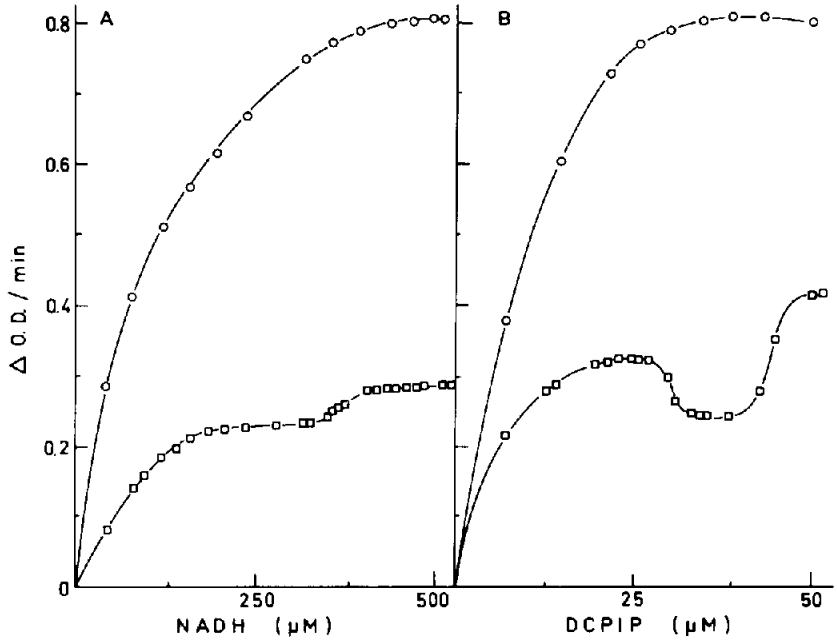

Fig.3. Effects of pretreatment of the enzyme with 10 μM BSA on the V vs $[S]$ plots. (□—□) control, (○—○) enzyme pretreated with BSA. (A) NADH as varying and 32 μM DCPIP as fixed substrate. (B) DCPIP as varying and 800 μM NADH as fixed substrate.

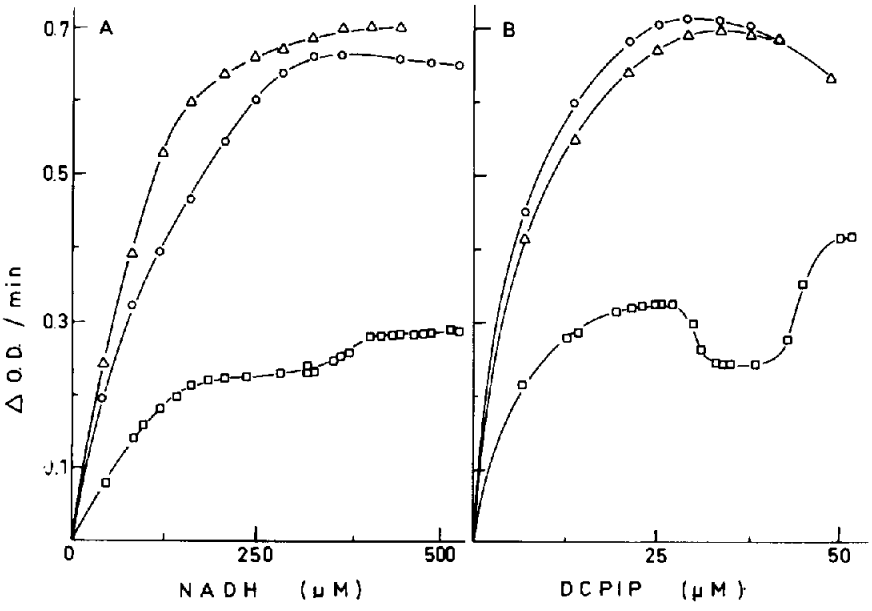

Fig.4. Effects of pretreating the enzyme with very large (> 1 000 000) modifiers on the V vs $[S]$ curves. (□—□) control, (○—○) pretreatment with a dispersion of sonically irradiated 1 mM egg-yolk lecithin, (△—△) pretreatment with 1 mg/ml of glycogen. (A) NADH as varying and 32 μM DCPIP as fixed substrate. (B) DCPIP as varying and 800 μM NADH as fixed substrate.

4. Discussion

The effect of pH and temperature on the V vs [S] plots of DT-diaphorase has been shown previously [4]. The use of these nonspecific effectors of protein conformation resulted in changes in the V vs [S] curves. Some low mol. wt substances such as ethanol and sucrose had the same effect (Hollander, unpublished results).

The high mol. wt substances used in the experiments above can be divided into two groups; one having a size similar to that found for DT-diaphorase (approx. 50 000) [9] and the other with mol. wt above one million. The sensitivity of the enzyme to compounds above a certain mol. wt is shown by the inefficiency of the Triton X-67, -305, -405 which form small micelles (mol. wt $< 10\,000$) [6]. On the other hand Triton X-100 and lysolecithin above their critical micellar concentrations and both having a micellar weight of about 85 000 [6,10] as well as BSA (mol. wt $\approx 67\,000$) had marked effects on the kinetics. Preliminary experiments also indicate that various dextranes (mol. wt = 70 000– 204 000) can abolish the plateau and 'trough' regions in the V vs [S] plots whereas dextrane (mol. wt = 20 000) has no effect on the curve shapes (Hollander, unpublished results). These results clearly indicate that the modifiers must have a minimum molecular size that is of the same magnitude as that found for DT diaphorase ($\sim$ 55 000, [9,11]). An exception to this was ethanol and sucrose. The basis for the effects of modifiers with mol. wt over 60 000 may be an interaction between the enzyme and the effector leading to the stabilization of one of the two enzyme species present. It was suggested in a previous report [4] that the basis for the complex V vs [S] curves is that two enzyme species in slow isomerization equilibrium with each other are present.

Ernster et al. [1] found that the enzyme was activated by BSA. Hosoda et al. [9] suggested that this effect of BSA is due to a specific facilitation of the dissociation of the reduced acceptor from the enzyme. The chemical nature of the modifiers (proteins, carbohydrates, lipids), however, is so varying that it is unlikely that they all possess specific binding sites for the reduced electron acceptor. The results of the pretreatment with different modifiers (i.e. results identical with those when the modifier was present in the assay mixture) also suggest that the effects of effectors are on the level of the enzyme structure and not on the substrates or products.

Acknowledgements

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