KINETIC STUDIES ON THE MECHANISM OF THE ACTION OF ADP ON THE GLUTAMATE DEHYDROGENASE REACTION*

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1. Introduction

ADP is known to activate the glutamate dehydrogenase (GluDH EC 1.4.1.3) reaction above pH 7 [2,3] whereas below this pH ADP is inhibitory. Therefore, it has been postulated that at low pH values, ADP instead of binding to an activator site binds to the inhibitor site which at high pH values is used by GTP [4]. Since NAD^+ shows a strong self-activating effect [5], the activation by ADP is dependent on NAD^+ concentration. The extrapolation of previous experimental data suggested that ADP was inhibitory at high NAD^+ concentrations [2]. However, before a general model for the ADP effect can be constructed, experiments must be performed to measure the ADP effect at low NAD^+ concentrations and to make a detailed investigation of the pH dependence of this reaction.

The data presented in this paper show that at all pH values, ADP has two effects: i) Increase of V_{max} with a simultaneous increase of the Michaelis constant for NAD^+. This effect causes activation at high and inhibition at very low NAD^+ concentrations. ii) Competitive inhibition with an inhibitor constant of about 6 mM which leads to an even further inhibition at low NAD^+ concentrations. The ratio of inhibition to activation increases with decreasing pH and increasing phosphate concentration. It is shown that all the observed effects can be explained on the basis of a single adenine binding subsite of the coenzyme binding site.

* Studies on glutamate dehydrogenase, part XI; for part X see [1].

2. Experimental

Beef liver GluDH (A_{280}/A_{260} ratio $\geqslant 1.95$) and the nucleotides were obtained from C.F. Boehringer and Soehne, Mannheim, Germany. All the other chemicals were of reagent grade purchased from E. Merck, Darmstadt, Germany. The reaction velocities were measured in an Eppendorf filter photometer (Eppendorf Gerätebau, Netheler und Hinz, Hamburg) equipped with a Vitatron Digilog logarithmic printer (Vitatron, Dieren, Holland), triggered by a timer constructed in our electronics division (time intervals between 3 and 30 sec, precision better than 1%). Cuvettes with light paths of 1 and 4 cm were used and the temperature stability was better than $\pm 0.1°$.

To eliminate the trivial first order curvature caused by the decrease of NAD^+ concentration during the reaction, we plotted log $([NAD^+_t]/[NAD^+_0])$ where $[NAD^+_t]$ is $[NAD^+_0]-[NADH]$; $[NAD_t]$ and $[NAD^+_0]$ are the NAD^+ concentrations at time t and zero time, respectively, versus time. With this plot an extrapolation to $[NADH] = 0$ was feasible by means of a least square parabola. The remaining curvature of the reaction curves is mainly caused by the NADH inhibition and will be discussed elsewhere. Second order correction (for substrate concentration) and backward reaction are irrelevant at the substrate concentrations used here.

3. Results and discussion

With ADP no inhibition could be observed at pH 8 even at very high NAD^+ concentrations. Therefore, the

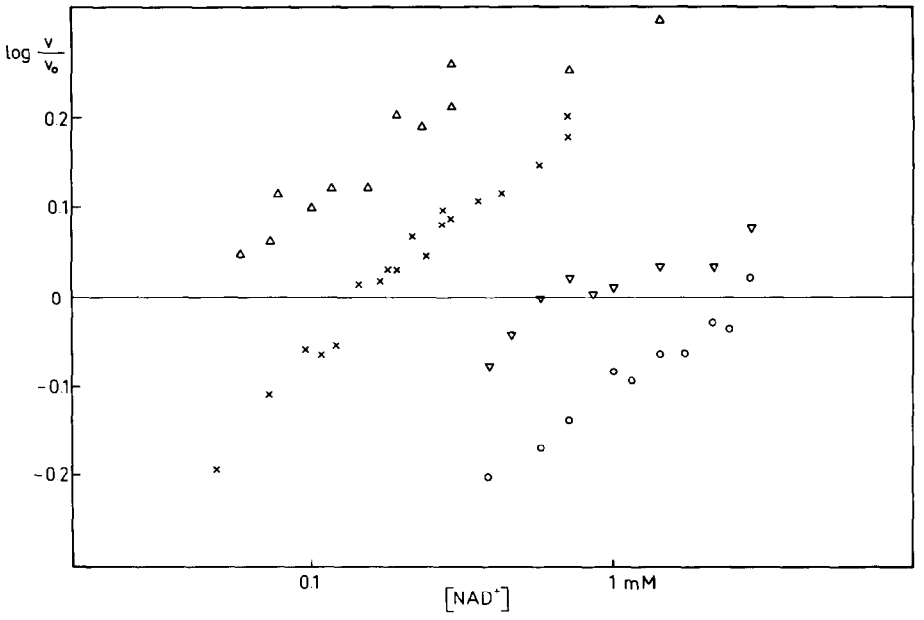

Fig. 1. Dependence of the ADP effect on NAD^+ concentration at different pH values. Measurements in 0.067 M sodium phosphate buffer at pH 7.0 (○), 7.3 (▽), 7.6 (×), and 7.9 (△) and 20°. Glutamate 8.5 mM, ADP 1 mM, GluDH 2.5 μg/ml, v is the initial velocity in the presence and v_0 in the absence of the effector.

linear extrapolation from the results obtained at low NAD^+ concentrations [2] in the presence of ADP does not appear to be justified.

Fig. 1 shows that the activating effect of ADP decreases with decreasing NAD^+ concentration. At pH 7.9 no inhibition was seen at the range of NAD^+ concentration we were able to use, but at pH 7.6 and 7.3 activation as well as inhibition was obtained and there is no doubt that also at pH 7.9 the data can be extrapolated into the inhibition region. On the other hand under the conditions of the experiment it was difficult to reach the activation region at pH 7.0. With constant ADP concentration the crossover point is clearly shifted to a higher NAD^+ concentration with decreasing pH. Higher phosphate concentrations act in the same direction. At low phosphate concentrations up to 0.067 M at pH 7.6 the effect of ADP is almost independent of the phosphate concentration, whereas at higher phosphate concentrations the inhibiting effect of ADP increases. (The activation plot is shifted laterally to the right so that the crossover point moves from 100 μM to 300 μM NAD^+ at 0.2 M at pH 7.6.) The activity without effector shows a dependence on the phosphate concentration parallel to that of the ADP effect, and the influence of higher salt concentration increases with decreasing pH [6]. A similar effect of pH and salt concentration on the association of the enzymatically active subunit (oligomer) to higher associated particles is observed. Whereas at low salt concentrations the association constant increases with decreasing pH [7], there is a dissociating effect at high salt concentrations which increases at low pH [6]. Possibly the influence of higher phosphate concentration on both the activity and the association–dissociation equilibrium results from some minor conformational changes induced by solvation effects on the enzyme.

In addition we have studied the effect of ATP and the effect of both ADP and ATP with $NADP^+$ as coenzyme. The effect of ATP is qualitatively the same as that of ADP with the difference being that the log v/v_0 curve with 3 mM ATP is almost the same as with 0.1 mM ADP indicating that the difference between ATP and ADP can be explained on the basis of a much higher dissociation constant with the former [8]. The differences between NAD^+ and $NADP^+$ are very small both qualitatively and quantitatively.

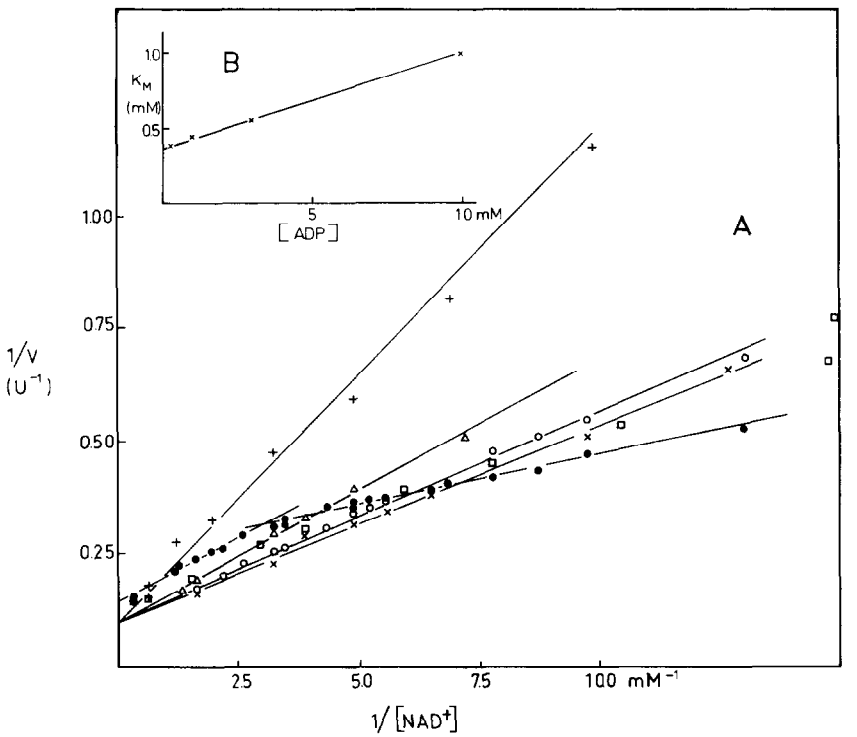

Fig. 2. A: Lineweaver-Burk plot for the reaction with NAD^+. Initial rate (v) measurements given in international units (U=μmoles/mg·min) in 0.067 M phosphate buffer, pH 7.6, at 8.5 mM glutamate and with ADP concentrations of 10 (+–+–+), 3 (△–△–△), 1 (○–○–○), 0.3 (x–x–x) and 0.025 mM (□–□–□), (●–●–●) is the control in the absence of ADP. B (Insert): Secondary plot of K_m (from A) K_m (from A) against ADP concentration.

If ADP and GTP would exchange binding sites with a change in pH, it should be possible to obtain activation by GTP under the same conditions under which ADP inhibits the reaction. However, even at pH 5.5 and NAD^+ concentrations down to 50 μM, strong inhibition by GTP is observed. Evidently there is no reciprocal relationship between ADP and GTP binding sites.

In order to get more insight into the ADP effect we studied the influence of a range of ADP concentrations at pH 7.6 (fig. 2). Whereas the control values exhibit one of the well-known discontinuities [9], (the others which were not seen are at lower NAD^+ concentrations than were used here) the plots with the higher ADP concentrations yield straight lines with the same V_{max} values which indicates that ADP is a competitive inhibitor with regard to NAD^+. Measurements with ADP concentrations less than 100 μM give values which show a smaller activating effect and thus differ from the above effect. Two effects must then be differentiated: i) An activation which under our conditions approaches saturation at 300 μM ADP and is much stronger than the NAD^+ self-activation. Table 1 shows that the ADP concentration for 50% of the maximum activation under our conditions at very high NAD^+ concentrations is about 30 μM. Simultaneously the Michaelis constant for NAD^+ increases, causing an inhibition at very low NAD^+ concentrations. ii) Competitive inhibition with respect to NAD^+ (K_I = 6 mM). In the secondary plots (fig. 2B) a Michaelis constant for NAD^+ of 360 μM was obtained for theoretical conditions where the inhibiting effect of ADP disappears without activation. Since the inhibition constant of 6 mM for ADP is very high compared with its dissociation constant which is about 2 μM [10,11] a direct competition with the adenine moiety of the NAD^+ molecule cannot be

Table 1
Dependence of the activation on the ADP concentration at pH 7.6 (0.067 M phosphate buffer) with 3.2 mM NAD^+ and 8.5 mM glutamate.

ADP (μM)	Activity (units)	v/v_0
0	5.94	–
25	7.15	1.21
50	7.82	1.32
100	8.43	1.42
Theoretical limit for $[ADP]_\infty$ without competition (from fig. 2B)	9.0	1.50

responsible for this effect. A less specific competition with other coenzyme binding subsites might be possible (e.g. for the nicotinamide moiety, cf. [10]). Since, on the other hand, it is most probable that ADP is bound to the enzyme at the adenine subsite of the coenzyme binding site, ADP might have two effects assuming a random order mechanism [9] for the reaction: First the dissociation of NADH from the final complexes is accelerated by ADP [12]. This could be due to competition at the adenine binding subsite. A mechanism involving the displacement of the adenine moiety of NADH by ADP followed by the dissociation of the nicotinamide moiety and finally the dissociation of ADP can be faster for entropic reasons than the dissociation of NADH in the absence of ADP. Secondly the competition of ADP with NAD^+ causes an increase of the Michaelis constant of the NAD by repression of all processes beginning with the binding of the coenzyme. But in those processes where the substrate, i.e. glutamate, binds first, the NAD^+ binding is perhaps tightened due to the binding of the dicarboxylic acid [13] so that much higher ADP concentrations are needed for competition with NAD^+. This could explain the very strong inhibition by ADP of the reaction with monocarboxylic acids where this tightening of NAD^+ binding does not occur [6]. A part of the pH dependence of the ratio inhibition to activation is simply due to the pH dependence of the absolute turnover number of the enzyme. But if we plot activation against activity there remains a real pH effect which might be caused by an increase in the binding of the coenzyme with increasing pH. The activation also increases with increasing glutamate concentrations because the turnover number increases and the reaction sequence starting with the substrate binding is favoured. Fig. 3 demonstrates that this

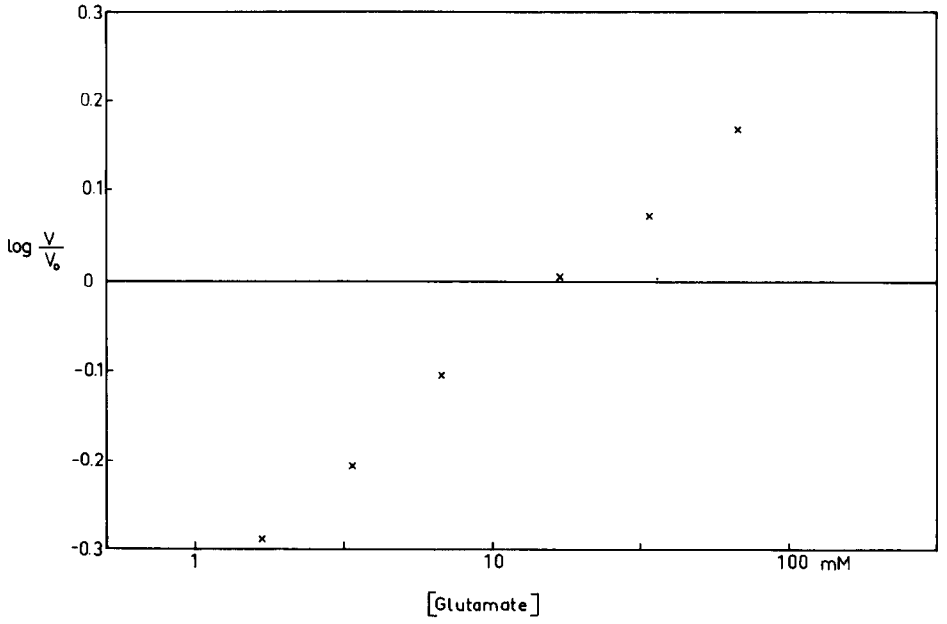

Fig. 3. Dependence of the ADP effect on glutamate concentration. Measurements in 0.067 M phosphate buffer at pH 7.6 and 20°, the reaction mixture contains 13.4 μM NAD^+, 500 μM ADP, 2.5 μg/ml GluDH.

dependence can be shown experimentally. Further investigations are in progress at very low coenzyme concentrations with fluorometric methods to get more insight in the first ADP effect.

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