REGULATION OF PIG HEART MITOCHONDRIAL GLUTAMATE DEHYDROGENASE BY NUCLEOTIDES AND PHOSPHATE: COMPARISON WITH PIG HEART AND BEEF LIVER PURIFIED ENZYMES

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1. Introduction

Previous work has shown the regulation exerted by Pi and adenylic nucleotides on glutamate oxidation and oxidative phosphorylation in pig heart mitochondria [1]. Since glutamate oxidation may be catalyzed to a large extent by GDH* (EC 1.4.1.3) [2, 3], it seemed important to study the direct effects of various nucleotides and Pi on GDH in pig heart mitochondria and to compare these effects with those exerted on purified enzymes from pig heart [4, 5] and from beef liver (commercial enzyme).

The main results are as follows: The three enzymatic systems can use NAD^+, $NADP^+$, deamino-NAD^+ and deamino-$NADP^+$ as coenzymes, but the regulatory properties of the effectors vary with the coenzyme.

ADP activation and GTP inhibition are of the same type in heart systems and in beef liver enzyme [6] when NAD^+ and $NADP^+$ are coenzymes. In the presence of deamino-NAD^+, the activation by ADP is abolished while GTP inhibition is not affected; ITP is as strong a negative effector as GTP. Pi enhances ADP activation and partly releases GTP inhibition with heart enzymes but not with the liver enzyme.

2. Materials and methods

Pig heart mitochondria were prepared according to Crane et al. [7] and washed, tested for respiratory control ratios, ADP/O, as previously [1].

Sonicated extracts S were prepared as follows: four sonications of twenty seconds each, separated by two minutes cooling, were applied to suspensions of washed mitochondria (30 mg proteins/ml, 0.25 M sucrose, 10 mM tris-HCl, pH 7.4, 0°) in an MSE disintegrator at maximum power. The sonicate was spun at 38,000 *g*, for 45 min in a Spinco preparative centrifuge, rotor 40, 4°. The supernatant S contained 95% minimum of glutamic dehydrogenase activity. Sonication could be replaced by a 0.1% Triton solubilizing treatment, in which case spinning was not neccessary; 0.1% Triton did not affect the activity while cholate could not be used to prepare such extracts since it inhibited the activity. However sonicates were preferred since centrifugation eliminates nucleotides bound to particulate fractions.

Purified pig heart enzyme was prepared according to Younes [4, 5].

Purified beef liver enzyme was purchased from Boehringer as a suspension in 50% glycerol. The nucleotides (purest qualities from Boehringer, P.L. Biochemicals, Inc. or Sigma Chemical Co.) were strictly tested by paper or thin layer chromatography on Gelman SA Silicagel, with isobutyric acid–aq. ammonia, specific gravity 0.88–water (66:1:33, v/v), to avoid contaminants.

Initial rates of GDH activity were determined at 340 nm by spectrophotometric measurements of reduced coenzyme after addition of the oxidized coenzyme; 5 mM KCN prevented any reoxidation of the coenzyme. The spectrophotometer allowed a precision of 0.001 absorbance units.

Proteins were estimated according to Weichselbaum [8].

In the kinetic studies, K_m and V_m were calculated

* GDH: glutamic dehydrogenase.

statistically by Wilkinson's method [9] and linearity tested by correlation coefficients.

3. Results

Fig. 1 shows variations of the initial rates of GDH activity in the pig heart mitochondrial supernatant S as a function of the concentration of various coenzymes. The activity is maximum with NAD, then decreases with the coenzymes in the following order: deamino-NAD, NADP and deamino-NADP. In a double reciprocal plot, the enzyme shows linear kinetics according to Michaelis and Menten with deamino-NAD or deamino-NADP as coenzyme; on the contrary, in the presence of NAD, enzyme kinetics deviate from linearity and apparently consist of two linear portions with different slopes, showing a downward bend within the physiological coenzyme concentration range. With NADP, the statistical calculations and the correlation coefficient (range 0.93 to 1) do not allow us to distinguish between linearity and a slightly biphasic curve. It will be shown in table 2 that the enzymes purified from pig heart or beef liver behave in the same way, provided that the incubation conditions are the same.

Table 1 shows that the actions of a series of nucleotide effectors on the initial rates of activity of the three types of GDH, measured in the presence of

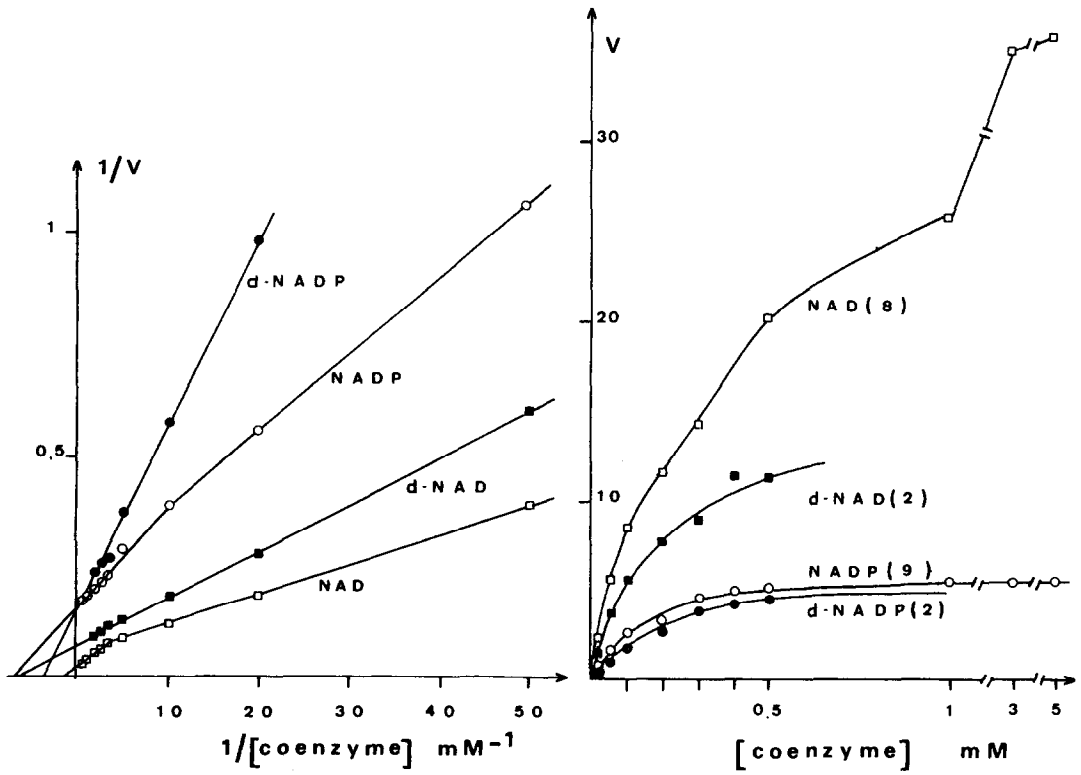

Fig. 1. Influence of coenzyme on the GDH velocity in pig heart mitochondrial extract. Supernatant S 1 mg protein (prepared according to Materials and methods). Medium (mM): tris-HCl 20, KCl 120, phosphate K 5, EDTA 1, KCN 5, glutamate K 25, pH 8.0, t = 28°. The reference cuvette is identical except that glutamate is omitted. The initial rate of GDH activity is measured at 340 nm after addition of coenzyme simultaneously in the two cuvettes. *V* is expressed in nmoles reduced coenzyme/100 sec/mg protein. d-NAD and d-NADP are, respectively, deamino-NAD and deamino-NADP. The curves are means of the number of experiments given in brackets.

Table 1
Compared role of various effectors on GDH activity with different coenzymes and different enzyme sources.

Enzyme	V'/V mitochondrial enzyme				V'/V purified heart enzyme			V'/V purified liver enzyme		
Coenzyme	NAD	NADP	d-NAD	d-NADP	NAD	NADP	d-NAD	NAD	NADP	d-NAD
N*	8	6	4	2	2	2	2	2	2	2
ATP	0.96	1.07	0.95	0.98	1.05	1.05	0.94	0.96	1.05	1
ADP	1.79	1.93	0.85	1.08	1.55	1.90	1	1.72	1.94	0.97
AMP	1.37	1.58	0.90	1.1	1.46	1.60	1	1.5	1.66	1.06
GTP	0	0	0	0	0	0	0	0	0	0
GDP	0.11	0.08	0.04	0.03	0.07	0.12	0.09	0.12	0.07	0.05
GMP	0.83	0.96	1		0.97	1	1			
ITP	0.09	0.05	0.02	0	0.06	0.03	0.04	0.16	0.12	
IDP	0.85	0.84	1		0.92	0.90	1.1			
IMP	0.89	0.98	0.96		0.94	1	0.9			
XTP	0.83	0.70	0.82	1						
XDP	0.92	0.9	0.92							
XMP	1	1	1.05							
other effectors**	~ 1	~ 1	~ 1		~ 1	~ 1	~ 1			

Conditions are the same as in fig. 1. Enzyme is 3×10^{-3} I.U.B. units in all cases. V is the rate in the absence of effector, V' is the initial rate in the presence of 0.2 mM effector. GDH activity is measured at 340 nm after addition of 0.2 mM coenzyme.

* N is the number of experiments.
** Other effectors studied: adenosine, adenine, hypoxanthine, xanthine, xanthosine, guanine, guanosine.

NAD, NADP, deamino-NAD or deamino-NADP, are of the same type. The number of phosphoryl groups seems very important for the action of the effectors: free bases and nucleosides have no effect; triphosphates have either no significant action (ATP) or are inhibitors. GTP and ITP inhibit very strongly while XTP is a poor inhibitor. Among the diphosphates, ADP is a powerful activator except if deamino-NAD is coenzyme; GDP is a strong inhibitor but less powerful than GTP (GDP contains no GTP as contaminant). On the other hand, the slight inhibition observed with IDP can be attributed to a 5% ITP contamination. AMP is a less efficient activator than ADP and GMP, IMP and XMP have no significant effect.

ADP is not an activator if the concentration of deamino-NADP is below 0.25 mM, but above this concentration an activation of 30 to 50% can be observed. On the other hand, ADP (0.2 to 1 mM) is not an activator at any deamino-NAD concentration (up to 1 mM), but is slightly inhibitory.

Fig. 2 illustrates the influence of Pi on NADP reduction by beef liver GDH. At the NADP concentra-

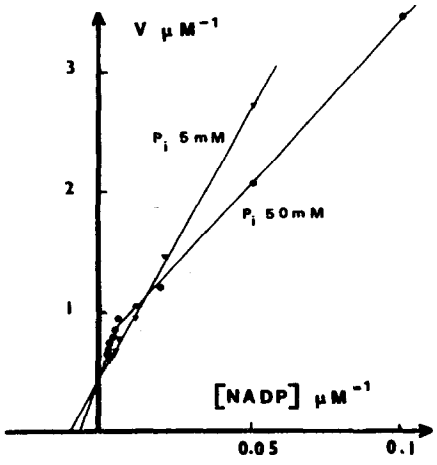

Fig. 2. Effect of Pi on beef liver GDH. Conditions as in fig. 1 except for assays with 50 mM Pi where KCl is omitted. Enzyme is 3×10^{-3} I.U.B. units. V is expressed as μmoles reduced coenzyme produced/minute/mg protein.

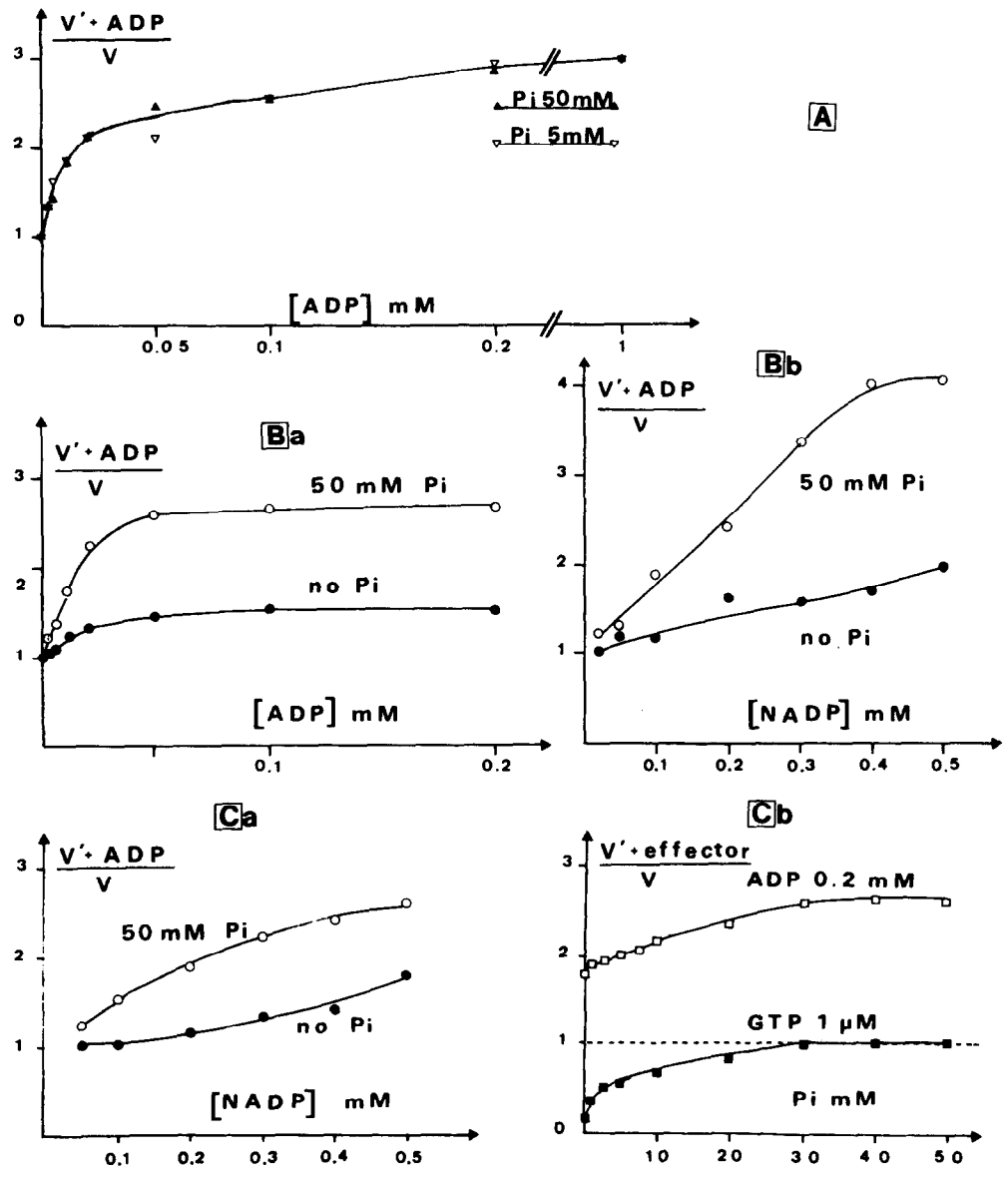

Fig. 3. Pi effects on ADP stimulation and GTP inhibition of GDH. Conditions as in fig. 2. (A) Effect of ADP concentration on beef liver GDH activity in the presence of 5 mM or 50 mM Pi with 0.2 mM NADP as coenzyme. V' is the rate in the presence of ADP; V is the rate without effector. (B) Effect of ADP and phosphate on purified pig heart enzyme. a) Same conditions as in A. b) V' is measured in the presence of 1 mM ADP. (C) a) Effect of ADP on mitochondrial pig heart enzyme. Conditions as in (B b) except that ADP is 0.2 mM. b) Effects of Pi concentration on 0.2 mM ADP activation and 1 µM GTP inhibition. Ionic strength is kept constant by modifying KCl concentration. NADP is 0.5 mM.

Table 2
Effects of Pi on V_m and K_m of beef liver GDH, purified pig heart GDH and mitochondrial pig heart GDH.

Enzyme	Coenzyme	Pi (5 mM)			Pi (50 mM)		
		Direct plot		Reciprocal plot Aspect*	Direct plot		Reciprocal plot Aspect*
		K_m app. (μM)	V_m app.		K_m app. (μM)	V_m app.	
Beef liver (commercial)	NADP	70	1.7	linear	50	1.7	biphasic
Purified pig heart	NAD	330	0.26	biphasic	300	0.185	biphasic
	NADP	79	0.061	∿ biphasic	59	0.045	biphasic
	d-NAD	122	0.115	linear	67	0.105	∿ biphasic
Mitochondrial pig heart (Supernatant S)	NAD	500	0.024	biphasic	348	0.023	biphasic
	NADP	120	0.006	∿ biphasic	52	0.005	biphasic
	d-NAD	150	0.015	linear	125	0.015	∿ bipjasic
	d-NADP	287	0.006	linear	–	–	–

Conditions as in fig. 2. The three different enzymes are prepared according to Materials and methods. Apparent K_m and V_m are determined on the direct plots of V versus coenzyme concentration.

* Aspect of the reciprocal plots giving $1/V$ versus 1/[coenzyme].

tion range studied, a plot of of $1/V$ versus 1/[NADP] appeared linear at 5 mM Pi, while two linear portions were observed with a downward bend at 50 mM Pi, ionic strength being kept constant.

Table 2 shows the effects of Pi on the apparent K_m and V_m measured for the three types of enzymes using the four coenzymes. With the two purified enzymes, the K_m and V_m values appear to be the same as those measured in 5 mM Pi, if Pi, KCN or EDTA is omitted, provided pH and ionic strength are the same. In all cases, increasing the Pi concentration up to 50 mM slightly lowers the V_m and K_m, except for the beef liver enzyme V_m; high Pi concentrations always affect the double reciprocal plot obtained: either linear variations become biphasic (liver enzyme) or the biphasicity is accentuated. On the contrary, 1 mM ADP abolishes biphasicity.

Fig. 3 shows the effects of Pi on ADP activation and GTP inhibition of the three glutamate dehydrogenases using NADP as coenzyme.

With purified beef liver enzyme, activation by ADP is totally independent of the Pi concentration (fig. 3A). On the contrary, with purified heart enzyme and mitochondrial heart enzyme, ADP activation is strongly enhanced by Pi; there is a cooperativity between Pi and NADP, since Pi stimulation depends on NADP concentration as shown in fig. 3B (a and b) and C (a and b). Fig. 3 C (b), shows that, without Pi, 1 μM GTP completely inhibits the mitochondrial enzyme and this inhibition is totally released if 50 mM Pi is added.

4. Discussion and conclusions

In the absence of Pi, the regulation of pig heart GDH (mitochondrial or purified) by nucleotides is the same as that observed with the purified liver enzyme: ADP and AMP are activators when NAD and NADP are coenzymes and GTP, ITP and GDP are inhibitors; the presence of Pi stimulates ADP activation only with heart enzymes and releases GTP inhibition.

The three enzymes (heart or liver) show no sigmoidal relation between velocity and coenzyme concentration; the downward bend observed in reciprocal plots in the presence of NAD of NADP, which is not expected from the Monod, Wyman, Changeux's model [10], has been interpreted by Dalziel [11], in the case of the liver enzyme, in terms of negative homotropic interactions between several identical active sites. Our

results show that the presence of Pi increases, or makes apparent, a biphasic curve with a downward bend, which could mean that Pi increases the negative homotropic interaction.

The fact that high concentrations of ADP tend to abolish the biphasic response towards coenzyme concentration in heart as in liver [6] and the fact that ADP activation is increased by Pi suggest that ADP releases the negative homotropic interactions. This interpretation is in agreement with the increase of the downward biphasicity observed in the presence of inhibitors, GTP or ITP. The relief of GTP inhibition by a high Pi concentration suggests that Pi diminishes the enzyme affinity for GTP.

If the coenzyme is deamino-NAD, the negative interaction is suppressed or very weak, suggesting that the adenyl-NH_2 plays a role in the conformational changes produced by interaction of sites. Since ADP activation does not take place with deamino-NAD, and is weak with deamino-NADP, the adenyl-NH_2 also interferes with the ADP activation.

The special sensitivity of heart GDH towards phosphate is very interesting when considering the regulation of glutamate oxidation by heart mitochondria. Heart is a muscle requiring much energy; any need for energy results in ATP breakdown leading to an increased ADP and Pi concentration; this stimulates glutamate dehydrogenation, the respiratory chain, and, at the same time, it abolishes possible GTP inhibition due to α-ketoglutarate dehydrogenation. This is in agreement with the fact that, in heart mitochondria, the percentage of glutamate oxidized by transamination is much less in the presence of ADP, while GDH is strongly stimulated [3].

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