BBA 66596

SOME THERMODYNAMIC DATA ON D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTION UNDER OPTIMAL CONDITIONS

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(Received December 10th, 1971)

SUMMARY

The minimal values of activation enthalpy and activation entropy of glyceraldehyde-3-phosphate oxidation under optimal conditions are 10–12 kcal/mole and −11–−12.5 cal/mole per degree, respectively. The minimal value of activation enthalpy of D-glyceraldehyde oxidation is 5.5–7.5 kcal/mole and that of activation entropy about −38 cal/mole per degree.

The pH optimum of the reaction and the Michaelis constants of substrates do not change in the temperature range examined. However, the thermodynamic data of enzyme action may be affected by changes in the dielectric constant of water due to the change in temperature.

The inhibition by excess NAD or ATP increases the activation energy of glyceraldehyde-3-phosphate oxidation by 4±1 kcal/mole, whereas inhibition by excess phosphate does not change the activation energy of the reaction. The entropy change is increased by about 2.5 cal/mole per degree after inhibition by excess phosphate, whereas inhibition by ATP or excess NAD appreciably decreases the entropy change (by about 15 cal/mole per degree).

INTRODUCTION

To elucidate the mechanism of action of D-glyceraldehyde-3-phosphate dehy-, drogenase (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12), in addition to the kinetics of the enzyme[1–6], the energetic aspects, *i.e.* the thermodynamics, of the enzyme action should also be known. As a first approach, the analysis of initial velocity of glyceraldehyde-3-phosphate and glyceraldehyde oxidation under optimal conditions as a function of temperature was chosen.

Until now it has only been known that the activation energy of the glyceral-

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dehyde-3-phosphate or glyceraldehyde oxidation catalyzed by the yeast, rabbit muscle and fish muscle enzymes, measured in the presence of arsenate ion (irreversible reaction), is between 13 and 19 kcal/mole[7–9]. However, no data have been available about the reaction with phosphate ion (physiological substrate, reversible reaction). Our results suggest that the measured values of activation enthalpy and activation entropy are not artefacts caused by changes in the pH optimum of the reaction or in the K_m values of substrates, or by heat denaturation at elevated temperatures. The increase in activation energy and the changes in activation entropy of the reaction due to some inhibitors have also been determined.

MATERIALS AND METHODS

The experiments were performed with four times recrystallized swine muscle enzyme[10], by using D-glyceraldehyde-3-phosphate[11] (prepared from fructose 1,6-diphosphate (Reanal)) or D-glyceraldehyde (Fluka), NAD (Reanal) and phosphate ion as substrates. The enzymic activity was measured in a Hilger UVISPEK and Opton PMQ II spectrophotometers at 340 nm, as described earlier[3,4].

The pH of the reaction mixtures was checked with a Radelkisz precision pH meter at the temperature of the experiment. The temperature of assay mixtures was kept constant ($\pm$0.2 °C) by using thermostated cuvette holders connected to a Kutesz Ultrathermostat.

The apparent rate constant, k, was calculated from the initial velocity, by measuring the change in absorbance in the first 5 s after mixing the enzyme with substrates. The apparent maximum velocity rate constant, k', was determined by keeping two of the three substrates at constant and optimal concentration and extrapolating to infinite concentration of the third substrate. In this way apparent rate constants are obtained by dividing velocity with total enzyme concentration, since the reaction follows the rapid equilibrium mechanism[1,3,5].

RESULTS AND DISCUSSION

The Arrhenius plot of D-glyceraldehyde-3-phosphate dehydrogenase action is shown in Fig. 1.

The activation energy of glyceraldehyde-3-phosphate oxidation is 12.3 $\pm$ 1.2 kcal/mole, independent of pH, and that of glyceraldehyde oxidation is 7.5 $\pm$ 1.3 kcal/mole. Thus the activation energies measured in the presence of physiological substrate (phosphate) are somewhat lower that those measured with arsenate[7–9].

The pH optimum of enzyme activity does not change with temperature (Fig. 2).

It is to be noted that the pH optimum at 38 °C is probably an apparent optimum because the protein is rapidly denatured above pH 8.8.

The Michaelis constants of substrates as determined from individual Lineweaver–Burk plots also slightly change with temperature (Table I).

Heat denaturation of the enzyme during the assay of enzymic activity does not cause more than 2% error, since the first order rate constant of heat denaturation of the enzyme is $2.21 \cdot 10^{-1}\text{min}^{-1}$ at 1 μg/ml concentration at the highest temperature used (38 °C) under the conditions of enzyme assay (but in the absence of substrates,

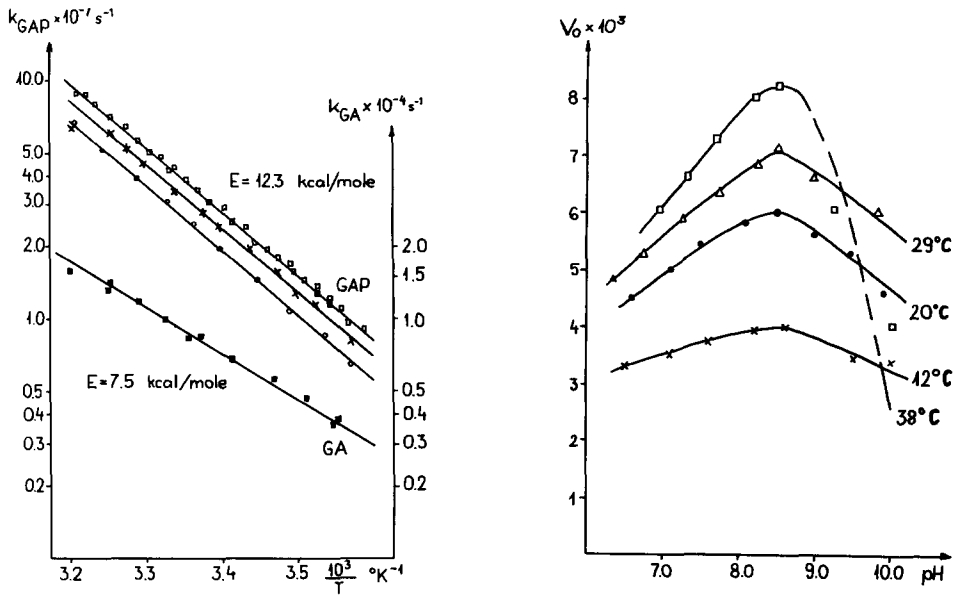

Fig. 1. Arrhenius plot of apparent rate constant as a function of the reciprocal of absolute temperature. k = apparent rate constant as defined in Materials and Methods, T = absolute temperature in °K. Assay mixture of glyceraldehyde 3-phosphate (GAP) oxidation: NAD, $2.1 \cdot 10^{-3}$ M; glyceraldehyde 3-phosphate, $2.3 \cdot 10^{-3}$ M; phosphate ion, $1.2 \cdot 10^{-2}$ M; enzyme, $6.9 \cdot 10^{-9}$ M; glycine buffer, 0.1 M. □—□, at pH 8.5; ○—○, at pH 6.0; ×—×, at pH 9.5. Assay mixture of glyceraldehyde (GA) oxidation: NAD, $2.1 \cdot 10^{-3}$ M; glyceraldehyde, $3.7 \cdot 10^{-2}$ M; phosphate ion, $1.2 \cdot 10^{-2}$ M; enzyme, $1.3 \cdot 10^{-6}$ M; glycine buffer, 0.1 M. ■—■, at pH 8.5.

Fig. 2. pH dependence of the rate of glyceraldehyde 3-phosphate oxidation at different temperatures. Assay mixture as in Fig. 1. v_0 = initial velocity in arbitrary units. The pH values were measured in the assay mixtures at the temperature indicated.

TABLE I

MICHAELIS CONSTANTS OF SUBSTRATES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AS A FUNCTION OF TEMPERATURE

The K_M values are calculated from individual Lineweaver–Burk plots at the given temperatures. Experimental conditions as given in legend to Fig. 3. Data for 20 °C taken from literature[12].

Temperature (°C)	*$K_m \times 10^4$ (M)*			
	Glyceraldehyde 3-phosphate	*Glyceraldehyde*	*NAD*§	*Phosphate*§
11–12	2.9	300*	1.6	4.2
20	5.0	500	1.2	2.0
23–25	3.1	500	2.3	2.4
38	2.2	300***	1.0	6.1
Average (independent of temperature and dielectric constant)	3.3	400	1.5	3.7**

* Measured at 15 °C.
** Depends on dielectric constant (unpublished results).
*** Measured at 35 °C.
§ Glyceraldehyde 3-phosphate was used as fixed substrate.

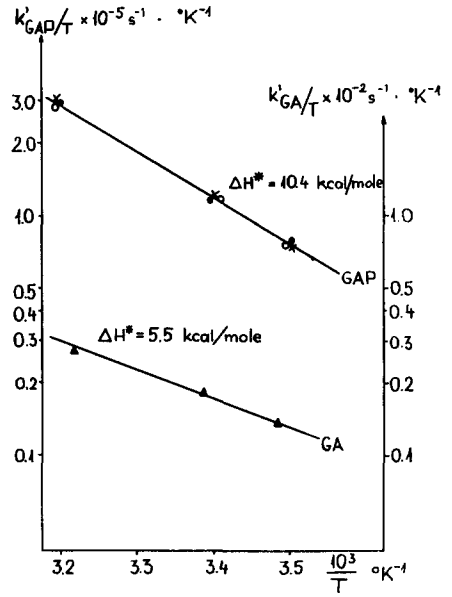

Fig. 3. Semilogarithmic plot of (k'/T) *vs* $1/T$. k' = apparent maximum velocity rate constant as defined in Materials and Methods. ×—×, data from saturation curves of glyceraldehyde 3-phosphate in the presence of $2.1 \cdot 10^{-3}$ M NAD and $1.2 \cdot 10^{-2}$ M phosphate ion. ○—○, data from saturation curves of NAD in the presence of $2.3 \cdot 10^{-3}$ M glyceraldehyde 3-phosphate and $1.2 \cdot 10^{-2}$ M phosphate ion. ●—●, data from saturation curves of phosphate ion in the presence of $2.3 \cdot 10^{-3}$ M glyceraldehyde 3-phosphate and $2.1 \cdot 10^{-3}$ M NAD. ▲—▲, data from saturation curve of glyceraldehyde, in the presence of $2.1 \cdot 10^{-3}$ M NAD and $1.2 \cdot 10^{-2}$ M phosphate ion. In each experiment 0.1 M glycine buffer, pH 8.5 was used.

which protect against heat denaturation), and initial velocity was calculated from the change in absorbance in the first 5 or 10 s.

The values of activation energy given above reflect true activation energies since neither the pH optimum of the reaction, nor the Michaelis constants of substrates change with the temperature. The activation energy determined from the Arrhenius plot of k (*cf.* Fig. 1) is in good agreement with that determined from the Arrhenius plot of the apparent maximum velocity constant (k', *cf.* Fig. 3, Table II), by using the equation:

$$k'/T = \kappa(R/Nh)\, e^{\Delta S^*/R}\, e^{-\Delta H^*/RT}$$

which in a semilogarithmic plot gives a straight line, the slope of which equals $-\Delta H^*/2.303R$ and the intercept with the ordinate equals $\log(R/Nh) + \Delta S^*/2.303R$, where ΔH^* and ΔS^* are the activation enthalpy and activation entropy, respectively, κ is the transmission coefficient assumed to be unity, R is the gas constant, N is the Avogadro number and h is the Planck constant (*cf.* ref. 13). The equality of activation energies obtained from Arrhenius plots of k and k' means that in both cases these values refer to the rate-limiting step.

The slow reaction rate of glyceraldehyde oxidation which differs by two orders of magnitude from that of the phosphorylated substrate may reflect the unusually high activation entropy. However, the difference in the reaction rates of glyceral-

TABLE II

ACTIVATION ENTHALPY AND ACTIVATION ENTROPY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTION

For experimental conditions see legend to Fig. 3. The concentration of the substrates listed were varied. ΔS^* was obtained extrapolating the plots of Fig. 3 to $1/T = 0$.

Substrate	*Temperature (°C)*	*k'* *($\times 10^{-3}$ s^{-1})*	*ΔH^* (kcal/mole)*	*ΔS^* (cal/mole per degree)*
NAD*	12.0	0.188		
	23.5	0.494	10.4	−11.1
	38.0	0.966		
Phosphate ion*	12.0	0.189		
	23.0	0.436	10.4	−11.2
	38.0	0.947		
Glyceraldehyde 3-phosphate	11.0	0.193		
	23.0	0.516	10.1	−12.5
	38.0	1.032		
Glyceraldehyde	15.0	0.0025		
	25.0	0.0031	5.5	−38.0
	35.0	0.0048		

* Glyceraldehyde 3-phosphate was used as fixed substrate.

dehyde and glyceraldehyde-3-phosphate oxidation may primarily be due to a difference in the transmission coefficients, which were arbitrarily assumed to be unity.

Our preliminary experiments show that the rate of glyceraldehyde-3-phosphate oxidation increases with increasing dielectric constant of the medium, and also the K_m of phosphate ion depends on dielectric constant. The dielectric constant of water decreases towards higher temperatures, whereas the apparent rate constant is increased. The change of apparent rate constant (k) due to changes of the dielectric constant of water at different temperatures and the change of apparent rate constant measured in media of different dielectric constants are opposite. Therefore it is reasonable to assume that the measured values quantitatively are the minimum values of activation enthalpy and entropy because of the partial compensatory effect of the decrease of dielectric constant of water by increasing the temperature. Of course, several other alterations may occur in the assay mixture by changing the temperature (changes in viscosity, water structure, *etc.*), however, the determination of their effect on enzyme activity are outside the scope of this paper.

It was of interest also to analyze the thermodynamics of the inhibited reaction in order to elucidate the energetical aspect of inhibition. It has been shown that excess of phosphate[14,15] (J. Ovádi, M. Nuridsány and T. Keleti, unpublished), NAD[6] and stoichiometric amounts of ATP[16] inhibit glyceraldehyde-3-phosphate oxidation. The function of inhibition by excess substrate[6] derived by plotting $1/[S]$ is constant, *i.e.* gives a straight line at high substrate concentration[17]. The intercept of this straight line with the ordinate gives the velocity at maximum inhibition ($v_{i,m}$). The optimum velocity is that measured in the presence of each substrate at optimal concentration (v'_m). Since

$$k = Ae^{-E/RT} \quad \text{and} \quad k_i = A_i e^{-E_i/RT}$$

where k and k_i are the rate constants, and A and A_i the pre-exponential factors and

E and E_i the activation energies of non-inhibited and inhibited reactions, respectively, multiplying with the total enzyme concentration (E_T) we obtain:

$$kE_T = AE_T e^{-E/RT} \quad \text{and} \quad k_i E_T = A_i E_T e^{-E_i/RT}$$

$$v'_m = A' e^{-E/RT} \quad \text{and} \quad v_{i,m} = A'_i e^{-E_i/RT}$$

$$\ln (v'_m/v_{i,m}) = \ln (A'/A'_i) + (E_i - E)/RT$$

$$\text{if } E_i - E = \Delta E$$

$$\lg (v'_m/v_{i,m}) = \lg (A^\circ) + \Delta E/2.303RT$$

The logaritm of $v'_m/v_{i,m}$ as a function of $1/T$ gives a straight line, the slope of which is directly related to the difference in activation energy between the uninhibited and inhibited reactions (Fig. 4).

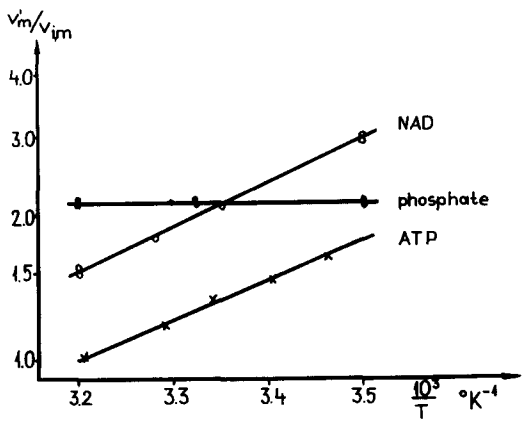

Fig. 4. Semilogarithmic plot of $v'/v_{i,m}$ *vs* $1/T$. The inhibition of enzymic activity with excess NAD[6], phosphate (J. Ovádi, M. Nuidsány and T. Keleti, unpublished) or with ATP[16] was performed as described earlier. Further explanations see in text. The slopes correspond to 4.5, 4.1 and 0 kcal/mole in the case of NAD, ATP and phosphate, respectively.

Inhibition by excess of NAD or by ATP causes an increase in activation energy of glyceraldehyde-3-phosphate oxidation of 4 ± 1 kcal/mole. Inhibition by excess of phosphate does not increase the activation energy. Applying this transformation to the equation of k', one can calculate the change in activation entropy caused by the inhibition. The inhibition by excess NAD and by ATP diminishes the change in entropy of glyceraldehyde-3-phosphate oxidation to a considerable extent (from about -12 to about $+3$ cal/mole per degree), whereas the inhibition by excess of phosphate changes the activation entropy from about -12 to about -14.5 cal/mole per degree. This approach gives a new method to determine the change in activation enthalpy and entropy by inhibition or activation, independent of the relatively high error in the determination of their absolute values.

Our data show that excess of phosphate does not inhibit by increasing activation energy, as do excess NAD or ATP, but through the increase in activation entropy. It has yet to be shown whether this phenomenon is related to the fact that phosphate ion participates in the rate-limiting step of the overall reaction.

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