BBA 66207

PROPERTIES OF MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM THE COLD-ADAPTED ANTARCTIC FISH *DISSOSTICHUS MAWSONI**

FRANK C. GREENE AND ROBERT E. FEENEY
Department of Food Science and Technology, University of California, Davis, Calif. 95616 (U.S.A.)

(Received June 15th, 1970)

SUMMARY

1. Muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) from the Antarctic cold-adapted fish *Dissostichus mawsoni* has been examined for evidence of cold adaptation at the molecular level. It has been found to be very similar to the rabbit muscle enzyme with regard to molecular size, amino acid composition, electrophoretic properties and pH activity profile. Similarity in the respective binding environments for the substrate glyceraldehyde 3-phosphate is suggested by similarity of the K_m values, susceptibility to substrate inhibition, reactivity with the specific acylating agent β-(2-furyl) acryloyl phosphate, and the effects of temperature on these characteristics.

2. There were large differences in the utilization of NAD^+ and its analogues. K_m values observed for the *D. mawsoni* enzyme with NAD^+, thio-NAD^+, 3-acetylpyridine adenine dinucleotide, and 3-acetylpyridine hypoxanthine nucleotide average about 5-fold greater than the corresponding values for the rabbit enzyme.

3. The activation energy of the glyceraldehyde 3-phosphate oxidation-NAD^+ reduction reaction as catalyzed by the *D. mawsoni* enzyme is 14 500 ± 1700 cal/mole, which is lower than the 18 100 ± 1200 cal/mole characteristic of the reaction catalyzed by the rabbit enzyme. The *D. mawsoni* has a higher specific activity near 0° than the rabbit enzyme. The *D. mawsoni* enzyme also was neither inactivated nor dissociated by ATP or AMP at low temperature, while the rabbit enzyme was inactivated and dissociated under the same conditions.

INTRODUCTION

In the process of adapting to lowered environmental temperatures, poikilothermic animals may experience metabolism shifts. For example, cold-acclimated trout show increased O_2 consumption and increased muscle and liver glycolytic and

* Parts of this material were taken from the thesis of F. C. G. in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry, University of California, Davis.

pentose phosphate shunt metabolism; and there occurs a consistent increase in total activity of all lactate dehydrogenase isozymes of brain, heart and muscle of cold-adapted goldfish[1,2]. Antarctic fishes are good examples of the effects of natural selection processes operating in response to life at low temperatures. This is especially true of the family Nototheniidae, many of whose members spend their lives at water temperatures near $-2°$ and who are obligatorily adapted to cold environments[3]. WOHLSCHLAG[4] has shown that native Antarctic fishes have higher O_2 metabolism than predicted from studies on more temperate fishes.

In previous studies of several cold-adapted Antarctic fishes, properties indicative of cold adaptation were found in the blood clotting mechanism[5], muscle aldolase[6], and the muscle and heart lactate dehydrogenases[7]. In the present study, results of a more detailed characterization of the properties of the muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) are reported. Glyceraldehyde-3-phosphate dehydrogenase was selected for this study because it is a key enzyme in glycolysis, representing the single oxidative step in the pathway[8-10] and because its composition and properties from a variety of species have been well characterized. Comparative studies by ALLISON AND KAPLAN[11], ALLISON AND HARRIS[12], and HARRIS AND PERHAM[13] suggest remarkable interspecies conservation of composition and primary structure. This provides a basis for using the well-defined rabbit muscle enzyme as a reference in the present studies.

MATERIALS AND METHODS

*D. mawsoni** *skeletal muscle*

Live fish (approx. 50 lb) were obtained during mid-December 1966 through holes in the ice sheet covering the $-1.9°$ waters of McMurdo Sound, Antarctica. The fish were bled, killed, and cut into sections; these were immediately frozen at $-20°$, shipped to Davis, Calif., at dry-ice temperature, and stored frozen at $-20°$. The glyceraldehyde-3-phosphate dehydrogenase used in this study was prepared from muscle sections of two of these fish.

Crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Sigma, Boehringer Mannheim, or prepared from frozen muscle by the method of FERDINAND[14]. β-(2-Furyl) acryloyl phosphate was a gift from Dr. Sidney A. Bernhard, University of Oregon. NAD^+, thio-NAD^+, 3-acetylpyridine adenine dinucleotide, 3-acetylpyridine hypoxanthine dinucleotide, *p*-nitroblue tetrazolium, phenazine methosulfate, glyceraldehyde 3-phosphate, and β-mercaptoethanol were obtained from Sigma Chemical Company. Other materials were commercially available reagent grade.

Enzyme purification

Glyceraldehyde-3-phosphate dehydrogenase from *D. mawsoni* was prepared by a modification of the procedure of ALLISON AND KAPLAN[11], substituting Sephadex gel

* *D. mawsoni* is a member of the family Nototheniidae, suborder Percoidea, order Perciformes. Other members of Percoidea include Black Bass, Sea Bass, Perch, and Snapper.

filtration for dialysis, and employing slightly different DEAE-cellulose chromatography conditions.

The purified enzyme was obtained as an amorphous precipitate, which was stored either as a moist paste or as a concentrated suspension (approx. 10 mg/ml) in the supernatant fluid. On two occasions, the amorphous suspension became crystalline on storage in the cold.

Enzyme activity

Glyceraldehyde-3-phosphate dehydrogenase assays were adapted from the method of CORI *et al.*[15]. The final reaction mixture (total volume 3.0 ml) included 0.03 M sodium pyrophosphate (pH 8.6), 0.5 mM glyceraldehyde 3-phosphate (determined by assay in excess NAD^+), 0.25 mM NAD^+, 5 mM sodium arsenate, 1 mM β-mercaptoethanol, and 1 mM EDTA. Reaction was initiated by the addition of 0.005 ml of enzyme solution with rapid mixing. The progress of the reaction ($\Delta A_{340\ nm}$) was monitored spectrophotometrically. Temperature was controlled to ± 0.1° in a jacketed cell holder with a circulating constant temperature bath. Specific activity is expressed as μmoles of NAD^+ reduced per min per mg of protein at 25°.

Effects of adenine nucleotides

The conditions described by CONSTANTINIDES AND DEAL[16] were used in studying the effects of ATP and AMP on enzyme activity. Enzyme aliquots (0.1 mg/ml) were incubated for 1 h at 0° with 0.1 M imidazole–0.1 M β-mercaptoethanol buffer (pH 7.0) containing ATP or AMP at concentrations from 1 to 18 mM. Then activity measurements were made at room temperature. To study the effect of ATP on quaternary structure, the enzyme was dialyzed overnight at 4° against the imidazole–β-mercaptoethanol buffer, containing 5 mM ATP, and sedimentation experiments performed at a protein concentration of 2 mg/ml and temperature of 5°.

Zone electrophoresis, activity staining

Cellulose acetate electrophoresis was done in a Beckman Microzone apparatus. Buffer was 0.1 M Tris, 0.001 M EDTA, and 0.001 M β-mercaptoethanol, titrated to pH 9.0 with 0.1 M glycine. Disc electrophoresis was performed according to DAVIS[17] with 0.001 M EDTA added. Enzyme activity was localized by the procedure of LEBHERZ AND RUTTER[18].

Apparent activation energies

Apparent activation energies were determined from the slopes of plots of log k *vs.* $1/T$ according to the integrated Arrhenius equation[19], where k = maximal initial rate of enzyme-catalyzed NAD^+ reduction as determined from plots of S/v *vs.* S (ref. 20). When measurements were to be made below 0°, the reaction buffer was made 17% (v/v) in ethylene glycol, which extends the solution freezing point to −6.7°. Appropriate controls with the ethylene glycol were done above 0°.

Acylation of glyceraldehyde-3-phosphate dehydrogenase with β-(2-furyl) acryloyl phosphate

The acylation and corresponding deacylations were done according to MAL-

HOTRA AND BERNHARD[21]. Each reaction progress curve was analyzed according to classical reaction kinetics[22].

Physical methods

The molecular weight of *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase was determined at 0.25% protein concentration in 0.05 M Tris (pH 9.0), 0.001 M β-mercaptoethanol, 0.001 M EDTA by low-speed short-column (approx. 2.7 mm) sedimentation equilibrium at 3° and 6166 rev./min using a Beckman Model E ultracentrifuge. High-speed sedimentation equilibrium measurements were made in 0.1 M Tris (pH 7.0), 0.001 M EDTA at speeds of 20 410 and 17 980 rev./min (after HOAGLAND AND TELLER[23]). Rayleigh interference optics were used. Molecular weight values were calculated according to the equation[24,25]:

$$\frac{\mathrm{d}\ln c}{\mathrm{d}r} = \frac{\omega^2 M(1 - \bar{v}\varrho)r}{RT}$$

The value of partial specific volume (0.730 cc/g) at 4° determined for rabbit glyceraldehyde-3-phosphate dehydrogenase by JAENICKE *et al.*[26] was used in these calculations.

Sedimentation coefficients ($s_{20,w}$) were determined from the slopes of plots of log x *vs.* t according to SCHACHMAN[27] at 59 780 rev./min in the 0.05 M Tris (pH 9.0), 0.001 M β-mercaptoethanol, 0.001 M EDTA buffer system. Protein concentrations were varied from 0.24 to 0.34%, and Schlieren optics were used.

Solutions' relative viscosities were determined by flow times in an Ostwald viscometer.

Amino acid analysis

The amino acid composition of the *D. mawsoni* enzyme was determined on 24-h, 110°, 6 M HCl hydrolysates, using the Technicon amino acid analyzer standard system. No corrections were made for destruction or incomplete hydrolysis.

Thermal stability of glyceraldehyde-3-phosphate dehydrogenase

Enzyme (4.5 μg) in 0.1 ml of 0.4 M $NaHAsO_4$ with or without 0.01 ml 0.006 M NAD^+ was incubated for 30 min at various temperatures, cooled to 0° in an ice bath, and assayed at 15° in the standard assay mixture.

RESULTS

Physical and chemical properties

Yield and activity on purification. The average specific activity of four *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase preparations was 50 μmoles of NAD^+ per min per mg under the standard assay conditions.

The yield of enzyme was approx. 300 mg/kg of muscle, or 3% of the extractable protein. Assays on crude muscle extracts indicate higher total dehydrogenase activity, based either on tissue weight or extractable protein.

Molecular size. Sedimentation coefficients ($s_{20,w}$) at 0.3% protein concentration of *D. mawsoni* enzyme were calculated as 7.65 S at 20° and 7.52 S at 3°. Molecular

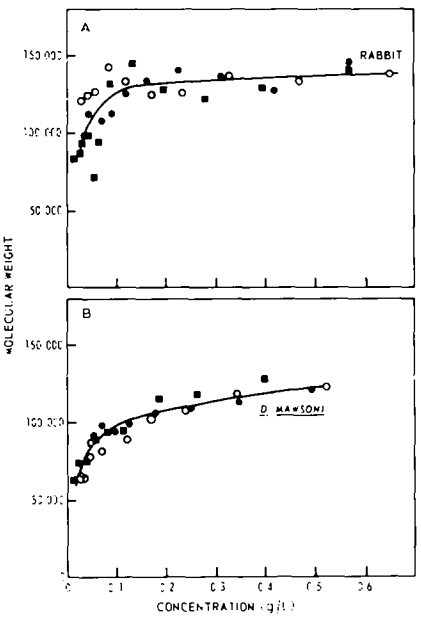


Fig. 1. Concentration dependence of glyceraldehyde-3-phosphate dehydrogenase molecular weight. A, *D. mawsoni* muscle glyceraldehyde-3-phosphate dehydrogenase with 1.5 moles NAD^+ per mole. B, Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with 1.8 moles NAD^+ per mole. Buffer, 0.1 M Tris 0.001 M EDTA (pH 7.0); Temperature, 4°. Initial protein concentrations are: ●, 0.75 g/l; ○, 0.50 g/l; ■, 0.25 g/l.

weight determined at 0.27% protein was approx. 142 000 by low-speed sedimentation equilibrium. The corresponding values at infinite dilution for rabbit muscle enzyme are: $s^0_{20,w}$ = 7.71 S (ref. 28) and mol. wt. 145 000 (ref. 29).

Results of high-speed equilibrium sedimentation experiments are given in Fig. 1. The enzyme from both rabbit and *D. mawsoni* muscle tends to dissociate at low concentrations, and the good overlap of the data from three different initial concentrations suggests dissociation equilibrium.

Multiple forms of the enzyme. When a crude extract of *D. mawsoni* muscle was subjected to cellulose acetate electrophoresis and stained for glyceraldehyde-3-phosphate dehydrogenase activity, four distinct bands were observed. A rabbit muscle extract showed only a single band when treated by the same procedure*.

Two chromatographic peaks of activity were resolved in the purification procedure. Disc gel electrophoresis, followed by stains for enzyme activity and protein, indicated one main component, with slight cross-contamination. The two peaks had similar specific activities over most of the elution range. The effect of temperature on reaction velocity was also similar for the two peaks, so the experimental work reported here was done with the two-isozyme mixture.

Amino acid composition of the enzyme. Table I gives the integral amino acid composition of muscle glyceraldehyde-3-phosphate dehydrogenase from *D. mawsoni*

* Separation by isoelectric focusing of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase into five peaks of activity has recently been reported[30].

TABLE I

APPROXIMATE AMINO ACID COMPOSITION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE SUBUNITS (RESIDUES PER 36 250 g)

Data obtained from: *D. mawsoni* (this study); halibut (ref. 40); rabbit (ref. 41); sturgeon (ref. 40, with exceptions of value for half-cystine from ref. 42); lobster and pig (ref. 13).

Amino acid	*D. mawsoni*	*Halibut*	*Rabbit*	*Sturgeon*	*Lobster*	*Pig*
Asp	39	44	38	39	32	30
Thr	21-22	18	24	20	20	22
Ser	19–20	22	21	18	25	19
Glu	20	23	20	20	24	18
Pro	13-14	13	13	13	12	12
Gly	30	29	34	29	30	32
Ala	35 36	32	32	34	32	32
Val	30–31	32	34	36	38	32
Cys	4		4	3	5	4
Met	9	9	9	9	10	9
Ile	19	22	18	20	18	19
Leu	17	19	19	19	18	17
Tyr	10	10	9	12	9	9
Phe	13–14	15	14	14	15	12
Lys	28	26	26	29	28	25
His	11	13	11	6	5	11
Arg	11	11	11	11	9	10
Trp	4	4	4	4	3	3

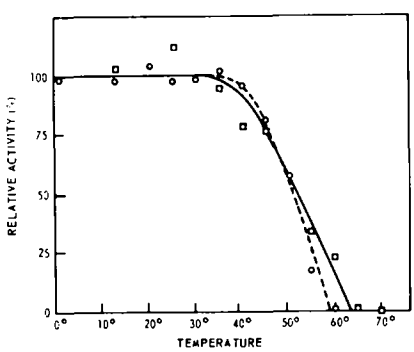


Fig. 2. Heat stability of rabbit and *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase. 4.5 μg enzyme were incubated for 30 min at indicated temperature, in the presence of 0.0006 M NAD^+, 0.4 M $NaHAsO_4$ (total volume 0.1 ml), immediately cooled to 0°, and assayed at 15°. □—□, rabbit; ○— —○, *D. mawsoni*.

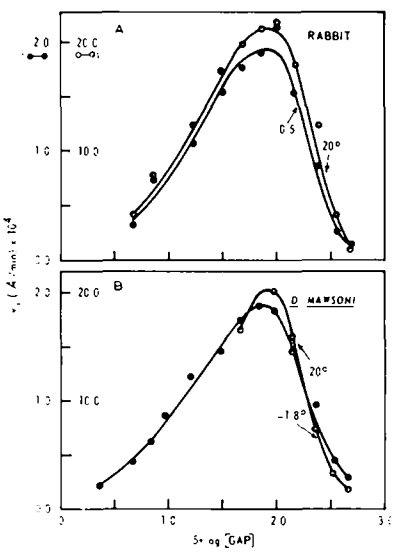


Fig. 3. Substrate inhibition of rabbit and *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase by glyceraldehyde 3-phosphate (GAP). Determination of K_i. Initial reaction velocity is plotted *versus* the log of the substrate concentration. The K_i values are estimated from the inflection points at the right limb of the curve. A. Rabbit: ○—○, 20°; ●—●, −0.5°. B. *D. mawsoni*: ○—○, 20°; ●—●, −1.8°.

and several other sources. In general, the enzymes are quite similar in composition.

Bound NAD⁺. The $A_{280\ nm}/A_{260\ nm}$ ratio of *D. mawsoni* indicated a bound NAD^+ content of about 4 moles/mole of enzyme, while the $A_{260\ nm}$ of the supernatant fluid after precipitation from 0.02 M $HClO_4$ indicated 3.2 moles NAD^+ per mole of enzyme. These values are similar to values of total[31] and enzymatically reducible[11] NAD^+ reported in the literature.

Heat stability. The relative activity remaining after 30 min at the indicated temperatures is shown in Fig. 2. The temperatures of 50% residual activity for the fish and rabbit enzymes are similar at 55°.

Catalytic properties

pH activity profile. Normalized (maximum activity defined as 100%) pH activity profiles of rabbit and *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenases at approx. 4° and approx. 20° were determined in 0.1 M Tris–0.004 M cysteine buffer; pH measurements were made at the temperature of the assay. The curves are quite similar below pH 9.0. An apparent inflection point occurs at approx. pH 7.5 for each enzyme, at both temperatures.

Substrate inhibition. Fig. 3 shows plots of $\log_{10}$ substrate concentration *vs.* initial velocity for the fish and rabbit enzymes, respectively, at moderate (20°) and low (1.8°) temperatures. Inhibition constants (K_i) determined from the inflection points are similar for the two enzymes at approximately 2 mM substrate. The K_i values show no apparent temperature dependence. Substrate inhibition of the rabbit enzyme by glyceraldehyde 3-phosphate has been reported at pH 7.4 by FURFINE AND VELICK[32].

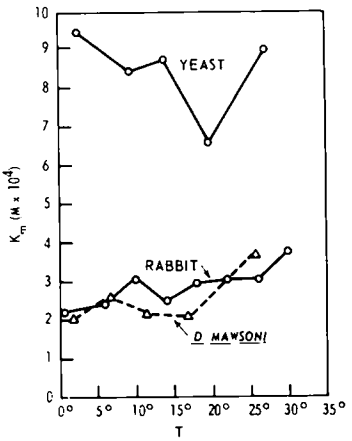
Reaction velocities, Michaelis constants, and their temperature relations. Relations of v_i to glyceraldehyde 3-phosphate concentrations were determined for glyceraldehyde-3-phosphate dehydrogenases from rabbit and *D. mawsoni* muscle and from yeast at several temperatures and 0.25 mM NAD^+. (The yeast data are included as representative of a distant species and as a test of mechanistic similarity.) Plots[20] of S/v *vs.* S, based on the equation $S/v = K_m/V + S/V$, yielded apparent V and K_m values.

The relation of K_m (substrate) to temperature is shown in Fig. 4. An additional K_m value for the *D. mawsoni* enzyme, measured in 17% ethylene glycol* at 2°, was 0.23 mM. The values for rabbit and *D. mawsoni* are similar in magnitude and in temperature dependence, showing an expected gradual increase with increasing temperature. The values for yeast are much higher, however, around 0.85 mM, and show a general different temperature trend.

Activation energies. Apparent activation energies (E_a) for the enzyme-catalyzed reaction were determined from Arrhenius plots of the maximal velocity data (Fig. 5). The fish enzyme has an E_a of 14 500 ± 1700 cal/mole, which is lower than the value of 18 100 ± 1200 cal/mole obtained for the rabbit enzyme**. It is also apparent from the Arrhenius plot that the maximal activity of the fish enzyme is similar to that of the rabbit enzyme at 25° but is approx. twice greater at lower temperatures. The E_a of the reaction with the yeast enzyme is approx. 13 400 cal/mole. Arrhenius plots were

* It has been reported by HERSKOVITS AND LASKOWSKI[33] that ethylene glycol does not structurally perturb proteins.

** The ionization state of the substrate is not likely to change significantly in this temperature range at pH 8.6, so it should not complicate the temperature dependence of the reaction.



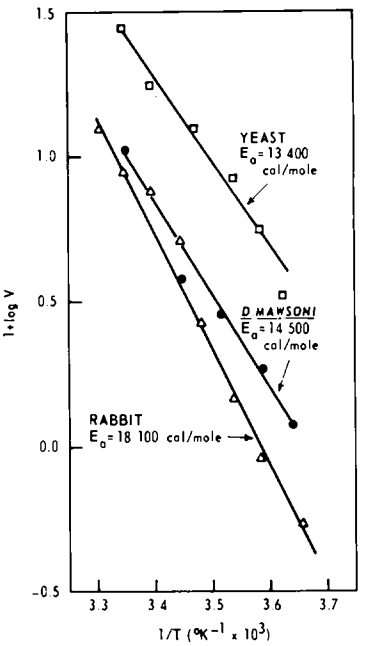


Fig. 4. Dependence of K_m (glyceraldehyde 3-phosphate) on temperature for rabbit muscle, *D. mawsoni* muscle, and yeast glyceraldehyde-3-phosphate dehydrogenase. Assay details as in text.

Fig. 5. Arrhenius plots for glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, *D. mawsoni* muscle, and yeast. Enzyme concentration approx. $2 \cdot 10^{-8}$ M, V in units of ΔA/min per mg. Assay details in text.

also made from data obtained at a lower substrate concentration (0.45 mM), which is closer to physiological concentration, and where the K_m values become influential in determining reaction rates and temperature dependences. These curves could be corrected to approximately straight lines if $\log_{10}[v(K_m + S_0)/S_0]$ (from rearrangement of the Michaelis–Menten equation) were substituted for $\log_{10} v$ in the plot with K_m values taken from data of Fig. 4. The slopes of these straight lines approximate those of the corresponding log V *vs.* $1/T$ lines in Fig. 5. Thus the temperature-activity relations observed at maximum velocity also prevail near physiological glyceraldehyde 3-phosphate concentrations.

Effects of adenine nucleotides. The effect of ATP and AMP on enzyme activity is shown in Fig. 6. The activity of the rabbit enzyme is reduced to 35% of the control value after incubation with 1 mM ATP and to only 16% of the control after incubation with 8 mM ATP. The *D. mawsoni* enzyme retains approx. 75% activity at 1 mM ATP and approx. 70% activity at 18 mM ATP. In addition, the *D. mawsoni* enzyme retains approx. 95% of control activity after incubation with AMP concentrations as high as 18 mM.

The dissociation of the rabbit enzyme by ATP at low temperature described by CONSTANTINIDES AND DEAL[16] was confirmed in sedimentation velocity experiments. A 0.2% solution of the rabbit enzyme in 5 mM ATP had two peaks with $s^{0.2\%}_{20,w}$ values of 7.47 and 4.43 S. Sedimentation in the absence of ATP yielded a single boundary with an $s^{0.2\%}_{20,w}$ value of 7.62 S. In contrast, the *D. mawsoni* enzyme gave only a single

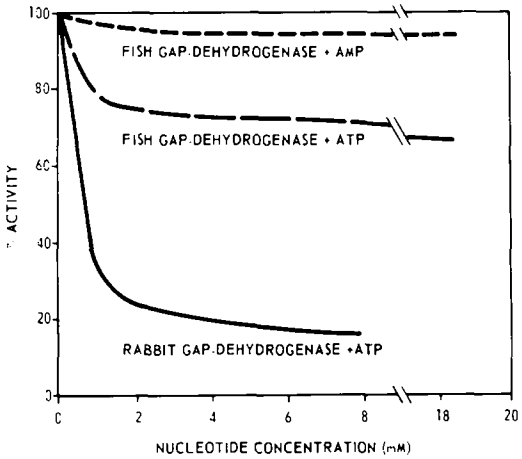


Fig. 6. Effect of adenine nucleotides on glyceraldehyde-3-phosphate dehydrogenase activity. Enzyme aliquots (0.1 mg/ml) were incubated for 1 h at 0° with the 0.1 M imidazole–0.1 M β-mercaptoethanol buffer (pH 7.0) and the indicated nucleotide concentrations. Activity measurements were then made at 25°.

boundary in the absence of ATP ($s^{0.2\%}_{20,w}$ = 7.70 S) and in the presence of ATP ($s^{0.2\%}_{20,w}$ = 7.58 S) (Fig. 7), confirming the retention of enzymatic activity in the presence of ATP.

Kinetics and stoichiometry of acylation of D. mawsoni glyceraldehyde-3-phosphate dehydrogenase by β-(2-furyl) acryloyl phosphate

Effect of NAD^- on the rate and stoichiometry of the acylation. According to MAL-

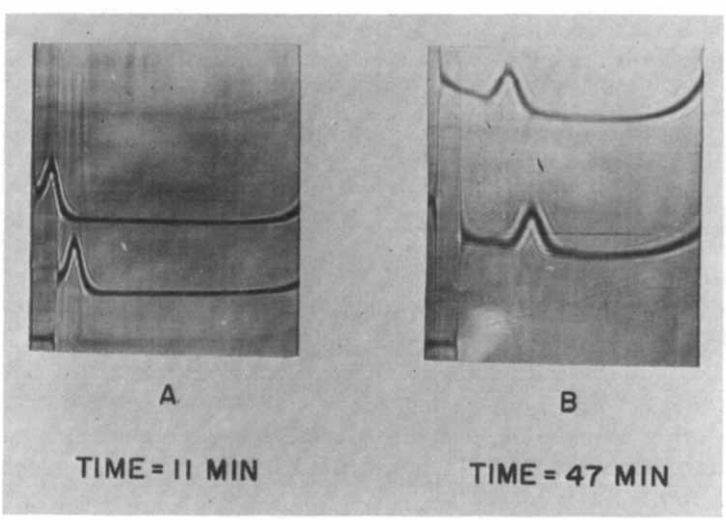


Fig. 7. Effect of ATP on sedimentation velocity of *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase. Enzyme was dialyzed overnight at 4° against the 0.1 M imidazole–0.1 M β-mercaptoethanol buffer (pH 7.0), with or without 5 mM ATP; then used at 2 mg/ml concentration in sedimentation velocity experiments at 5°. Upper pattern, with ATP, $s^{0.2\%}_{20,w}$ = 7.58 S; lower pattern, without ATP, $s^{0.2\%}_{20,w}$ = 7.70 S. Rotor velocity was 59 780 rev./min. Bar angles were 70° at 11 min and 50° at 47 min.

TABLE II

EFFECT OF NAD^+ CONCENTRATION ON STOICHIOMETRY AND RATE OF ACYLATION OF *D. mawsoni* AND RABBIT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Temperature 24°.

Glyceraldehyde-3-phosphate dehydrogenase	*β-(2-furyl)-acryloyl phosphate (μM)*	*Glyceraldehyde-3-phosphate dehydrogenase (μM)*	*$(NAD^+)^*$ / (Enzyme)*	*k acylation ($sec^{-1} \times 10^3$)*	*Moles acyl group bound per mole enzyme*
D. mawsoni	3900	7.23	1.0	1.12	1.66
	3900	8.50	3.3	2.58	1.81
	3900	7.53	5.5	3.55	2.07
Rabbit muscle	3700	7.65	1.7	3.08	1.73
	3700	8.33	1.8	3.02	1.82

* Molar ratio.

HOTRA AND BERNHARD[21] β-(2-furyl) acryloyl phosphate acts to acylate active site sulfhydryls of glyceraldehyde-3-phosphate dehydrogenase, causing, in the rabbit muscle enzyme, a perturbation of the furylacryloyl λ_{max} from 307 to 344 nm.

Results very similar to these were obtained with the *D. mawsoni* enzyme, which reacts at a pseudo first-order rate to form an acyl enzyme with a characteristic absorption maximum at 345–346 nm. Some rate constants and reaction stoichiometry data are given in Table II. Both reaction parameters are directly related to the NAD^+/enzyme ratio, in general agreement with the rabbit glyceraldehyde-3-phosphate dehydrogenase report. Also shown in Table II are results of acylation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. These rates are comparable to those reported by MALHOTRA AND BERNHARD[21]. Differences in the acylation rates of the two enzymes may reflect different affinities for NAD^+.

The reactions assume a higher order of enzyme concentration dependence at −3°, but their reaction rates appear similar.

TABLE III

EFFECT OF TEMPERATURE ON RATES OF DEACYLATION OF *D. mawsoni* AND RABBIT ACYL GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES BY ARSENATE

Source of glyceraldehyde-3-phosphate dehydrogenase	*AsO_4 (μM)*	*Acyl glyceraldehyde-3-phosphate dehydrogenase (μM)*	*Temp.*	*k deacylation ($l \cdot mole^{-1} \cdot sec^{-1}$)*
D. mawsoni	64 000	2.50	24°	3060
	65 300	2.84	14°	750
	66 300	2.39	2°	270
Rabbit	66 600	3.17	25°	5210
	66 600	2.78	14.8°	1440
	66 600	1.27	0.7°	405

TABLE IV

UTILIZATION OF NAD^+ AND NAD^+ ANALOGS BY GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Temperature, 25°.

Coenzyme	*Apparent maximal velocity at 0.5 mM glyceraldehyde 3-phosphate (μmoles coenzyme/min per mg protein)*		*Relative maximal velocity*		*Apparent K_m × 10^5 (M)*		*Ratio of apparent K_m values (D. mawsoni/rabbit)*
	Rabbit	*D. mawsoni*	*Rabbit*	*D. mawsoni*	*Rabbit*	*D. mawsoni*	
NAD^+	22.4	31.9*	100.0	100.0	1.80	7.02	4.2
Thio-NAD^+	2.4	2.3	9.3	7.2	4.35	23.5	5.4
3-Acetylpyridine adenine dinucleotide	1.0	0.9	4.5	2.8	15.8	62	3.9
3-Acetylpyridine hypoxanthine nucleotide	0.7	0.4	3.2	1.3	143	~815	~5.7

* This preparation of enzyme had been stored for some time and had a slightly lower specific activity than most preparations.

Deacylation rates. As previously noted[21], the deacylation does not follow simple first-order kinetics. Rates obtained from second-order (acyl enzyme) plots are shown in Table III. The deacylated enzyme had approx. 70% of its original activity. The deacylation of the rabbit β-(2-furyl)acryloyl enzyme is faster at each temperature than its *D. mawsoni* counterpart. However, the dependence of deacylation rate on temperature is similar for the two acyl enzymes.

Enzyme activities with NAD^+ and NAD^+ analogues. Michaelis constants and apparent maximal reaction velocities were determined for *D. mawsoni* and rabbit muscle glyceraldehyde-3-phosphate dehydrogenases utilizing NAD^+ and three of its analogues (Table IV). For a given coenzyme, the corresponding V values with the two enzymes are relatively similar, in contrast to the large differences between corresponding apparent K_m values (the K_m values probably correspond to the enzymes' most weakly binding NAD^+ sites). In each case, the larger apparent K_m is characteristic of the *D. mawsoni* enzyme, averaging about 5 times greater than for rabbit enzyme.

DISCUSSION

A comparison of the *D. mawsoni* and rabbit glyceraldehyde-3-phosphate dehydrogenases has shown the fish enzyme to have several characteristics favoring its functioning at low temperatures. The activation energy of the reaction of *D. mawsoni* glyceraldehyde-3-phosphate dehydrogenase has been measured as 14 500 ± 1700 cal/mole, and that of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase as 18 000 ± 1200 cal/mole. The difference is of thermodynamic significance. A low activation energy could be advantageous in a cold-adapted enzyme, for example, because it implies that a larger proportion of the enzyme molecules would be in an

activated state at a given temperature than would those enzymes with a high activation energy. Consistent with this is a higher specific activity of the fish enzyme than that of the rabbit enzyme at low temperature. Activation energy measurements by COWEY[34] of 19 000 cal/mole for rabbit muscle and 14 500 cal/mole for lobster and cod agree with these conclusions.

Furthermore, in the presence of ATP at low temperatures, the *D. mawsoni* enzyme is much less sensitive to inactivation and dissociation than is the rabbit enzyme. This property of resistance to inactivation would also be advantageous for the fish. A similar relative insensitivity to ATP has recently been reported for glyceraldehyde-3-phosphate dehydrogenase of honeybees* which are exposed to low temperatures during hibernation. The differential perturbation of the rabbit and *D. mawsoni* enzymes by ATP persisted unchanged to the highest protein concentration (2 mg/ml), whereas sedimentation equilibrium experiments at concentrations as low as 0.25 mg/ml in the absence of ATP failed to detect differences. The reactivities of the two enzymes with NAD^+ and NAD^+ analogs also indicate structural differences which may possibly be related to the difference in ATP effect.

No significant differences between the two enzymes are indicated by the physicochemical studies. Similarities revealed by the acylation–deacylation experiments, the susceptibilities to substrate inhibition, and K_m–temperature dependencies indicate a remarkable degree of identity in the active site region of the two enzymes. HOCHACHKA AND SOMERO[35,36] and SOMERO[37] have suggested, based on studies of lactate dehydrogenase and pyruvate kinase from several poikilotherms, that enzymes of such animals become adapted to exhibit minimum substrate K_m values near the animals' environmental temperatures. Analogous K_m minima have been observed neither in the *D. mawsoni* enzyme nor in the poikilotherm aldolase study of KOMATSU AND FEENEY[6]. KAYNE AND SEULTER[38,39] have reported that a conformational change in rabbit muscle pyruvate kinase accompanied by change in activation energy may be mediated by change of temperature, pH, and concentration of activating cations. Similar alterations in poikilothermic pyruvate kinases might well be accompanied by K_m minima, and may not be of adaptive significance.

ACKNOWLEDGMENTS

The authors greatly appreciate the technical assistance of Mrs. Judy Tweedie and Stephen Chan, the assistance of John Bigler, Stanley K. Komatsu, James Moore, James G. Norris, David T. Osuga and Augusto Trejo-Gonzales who participated in the Antarctic phase of the program, and advice and criticisms of Prof. John R. Whitaker.

Presented in part at the 160th Meeting of the American Chemical Society, Chicago, Ill., September 13–18, 1970. Supported in part by Grant GA-12607 from the Environmental Sciences Division, National Science Foundation and by financial support of F. C. G. by the U.S. Department of Agriculture.

F. C. G. is an employee of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif. 94710.

* Published in abstract form: W. GELB AND J. H. NORDIN, *Abstr. Federation Am. Soc. Exptl. Biol.*, No. 2362 (1970).

REFERENCES

1 P. W. HOCHACHKA AND F. R. HAYES, *Can. J. Zool.*, 40 (1962) 261.
2 P. W. HOCHACHKA, in C. L. PROSSER, *Molecular Mechanisms of Temperature Adaptation*, Am. Assoc. Advan. Sci., Washington, D.C., 1967, p. 177.
3 G. N. SOMERO AND A. L. DEVRIES, *Science*, 156 (1967) 257.
4 D. E. WOHLSCHLAG, in M. L. LEE, *Biology of the Antarctic Seas, Antarctic Research Series*, Vol. I, American Geophysical Union, Washington, D.C., 1964, p. 33.
5 S. K. KOMATSU, H. T. MILLER, A. L. DEVRIES, D. T. OSUGA AND R. E. FEENEY, *Comp. Biochem. Physiol.*, 32 (1970) 519.
6 S. K. KOMATSU AND R. E. FEENEY, *Biochim. Biophys. Acta*, 206 (1970) 305.
7 R. E. FEENEY AND D. T. OSUGA, *Antarctic Biology*, Am. Assoc. Advan. Sci., Washington, D.C., in the press.
8 K. UYEDA AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 4689.
9 S. RAPOPORT, in P. N. CAMPBELL AND G. D. GREVILLE, *Essays in Biochemistry*, Vol. 4, Academic Press, New York, 1968, p. 69.
10 S. P. COLOWICK, J. VAN EYS AND J. H. PARK, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Vol. 14, Elsevier, Amsterdam, 1966, p. 1.
11 W. S. ALLISON AND N. O. KAPLAN, *J. Biol. Chem.*, 239 (1964) 2140.
12 W. S. ALLISON AND J. I. HARRIS, *Proc. 2nd Meeting Federation of European Biochem. Soc., Vienna, Austria, 1965*, p. 140.
13 J. I. HARRIS AND R. N. PERHAM, *Nature*, 219 (1968) 1025.
14 W. FERDINAND, *Biochem. J.*, 92 (1964) 578.
15 G. T. CORI, M. W. SLEIN AND C. F. CORI, *J. Biol. Chem.*, 173 (1948) 605.
16 S. M. CONSTANTINIDES AND W. C. DEAL, JR., *J. Biol. Chem.*, 244 (1969) 5695.
17 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
18 H. LEBHERZ AND W. J. RUTTER, *Science*, 157 (1967) 1198.
19 E. A. DAWES, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Vol. 12, Elsevier, Amsterdam, 1964, p. 89.
20 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 2nd ed., 1964, p. 145.
21 O. P. MALHOTRA AND S. A. BERNHARD, *J. Biol. Chem.*, 243 (1968) 1243.
22 W. J. MOORE, *Physical Chemistry*, Prentice Hall, Englewood Cliffs, 1962, p. 253.
23 V. D. HOAGLAND AND D. C. TELLER, *Biochemistry*, 8 (1969) 594.
24 T. SVEDBERG AND K. O. PEDERSEN, *The Ultracentrifuge*, Oxford University Press, London, 1940, p. 51.
25 K. E. VAN HOLDE, *Fractions*, No. 1, Beckman Instruments, Inc., Palo Alto, Calif., 1967, p. 1.
26 R. JAENICKE, D. SCHMID AND S. KNOF, *Biochemistry*, 7 (1968) 919.
27 H. K. SCHACHMAN, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959, p. 75.
28 J. B. FOX AND W. B. DANDLIKER, *J. Biol. Chem.*, 218 (1956) 53.
29 W. F. HARRINGTON AND G. M. CARR, *J. Mol. Biol.*, 13 (1965) 885.
30 W. A. SUSOR, M. KOCHMAN AND W. J. RUTTER, *Science*, 165 (1969) 1260.
31 A. C. MURDOCK AND O. J. KOEPPE, *J. Biol. Chem.*, 239 (1964) 1983.
32 C. S. FURFINE AND S. F. VELICK, *J. Biol. Chem.*, 240 (1965) 844.
33 T. HERSKOVITS AND M. LASKOWSKI, JR., *J. Biol. Chem.*, 237 (1962) 2481.
34 C. B. COWEY, *Comp. Biochem. Physiol.*, 23 (1967) 969.
35 P. W. HOCHACHKA AND G. N. SOMERO, *Comp. Biochem. Physiol.*, 27 (1968) 659.
36 G. N. SOMERO AND P. W. HOCHACHKA, *Biochem. J.*, 110 (1968) 395.
37 G. N. SOMERO, *Biochem. J.*, 114 (1969) 237.
38 F. J. KAYNE AND C. H. SEULTER, *J. Am. Chem. Soc.*, 87 (1965) 897.
39 F. J. KAYNE AND C. H. SEULTER, *Biochemistry*, 7 (1968) 1678.
40 N. O. KAPLAN, in V. BRYSON AND H. J. VOGEL, *Evolving Genes and Proteins*, Academic Press, New York, 1965, p. 243.
41 S. F. VELICK AND C. FURFINE, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 7, Academic Press, New York, 2nd ed. 1963, p. 243.
42 W. S. ALLISON AND N. O. KAPLAN, *Biochemistry*, 3 (1964) 1792.