BBA 66089

A HEAT LABILE FRUCTOSEDIPHOSPHATE ALDOLASE FROM COLD-ADAPTED ANTARCTIC FISHES*

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(Received December 29th, 1969)

SUMMARY

Muscle fructosediphosphate aldolases (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase; EC 4.1.2.13) from the cold-adapted Antarctic fishes *Trematomus borchgrevinki* and *Dissostichus mawsoni* were purified by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE- and CM-cellulose, and fractionation on Sephadex G-200. The fish fructosediphosphate aldolases were similar to rabbit muscle fructosediphosphate aldolase in terms of molecular size, electrophoretic properties, pH–activity profiles, specific activities, activation energies, and Michaelis constants at −2°. The fish fructosediphosphate aldolases, however, differed significantly from rabbit fructosediphosphate aldolase in that they were more temperature labile and contained only 16–17 sulfhydryl groups as compared to 28 sulfhydryl groups in rabbit fructosediphosphate aldolase. Reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 0° led to slow inactivation of the enzymatic activity of the fish fructosediphosphate aldolases and activity was completely lost when only about 9 sulfhydryl groups were reacted. In contrast, rabbit fructosediphosphate aldolase retained > 80% of its activity after 9 of its sulfhydryl groups were reacted. Reaction with DTNB at 25° led to rapid inactivation of the fish fructosediphosphate aldolases but not of the rabbit enzyme. At 25° all 16 sulfhydryl groups of the fish fructosediphosphate aldolases, but only 10 of the 28 sulfhydryl groups of rabbit fructosediphosphate aldolase, reacted with DTNB without the addition of sodium dodecyl sulfate.

INTRODUCTION

Several species of Antarctic fishes have been shown by WOHLSCHLAG[1-3] to be well adapted to the −1.86° environment of the sea water of McMurdo Sound, Antarctica. These fishes belong to the family Nototheniidae, and are almost entirely limited to Antarctic waters[4]. The Nototheniidae species *Trematomus borchgrevinki*,

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

* This material was presented by S.K.K. to the Graduate Division, University of California, as part of the requirements for the Ph. D. degree in Biochemistry.

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Trematomus bernacchii, and *Trematomus hansoni* are true poikiliotherms and have an upper temperature tolerance of 6° (ref. 5). We have been examining the blood and muscle proteins of these fishes in a study of the effects of cold adaptation on the molecular systems of higher organisms[6,7]. This paper presents a comparison of the physico-chemical and enzymatic properties of fructosediphosphate aldolases prepared from these fishes and from rabbit muscle.

Fructosediphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was discovered in muscle in 1934 by MEYERHOF AND LOHMANN[8]. Since then it has been identified in microorganisms and plants. Fructosediphosphate aldolases have been classified into two groups. Class I fructosediphosphate aldolases are present in higher animals, higher plants, protozoans, and green algae[9] and have a molecular weight of 160 000 (ref. 10), and are inhibited by Ag^+ and Cu^{2+}. Class II fructosediphosphate aldolases are present in bacteria, yeast, and blue green algae, have a molecular weight of 70 000, and are inhibited by metal chelating agents[11].

Most preparations of rabbit muscle fructosediphosphate aldolase are prepared by the crystallization method of TAYLOR *et al.*[12]. Early investigations indicated that rabbit muscle fructosediphosphate aldolase consists of three subunits. Recently, however, hybridization and centrifugation experiments have revealed that rabbit muscle fructosediphosphate aldolase has four subunits[10,13-17]. KOCHMAN *et al.*[17] have shown that native rabbit fructosediphosphate aldolase has a tetrameric structure composed of two different subunits which can be hybridized to give five isozymic forms. Residues of lysine[18], histidine[19], tyrosine[20], and cysteine[21-24] have been implicated as being essential for the catalytic activity.

It has been shown by many workers that enzymatic activity of rabbit muscle fructosediphosphate aldolase is lost when 10–14 sulfhydryl groups are modified by *p*-mercuribenzoate, *N*-ethylmaleimide, chlorodinitrobenzene[21], fluorodinitrobenzene[22], or tetranitromethane[23]. SWENSON AND BOYER[24] have shown that, in the presence of phosphate, 10 sulfhydryl groups of fructosediphosphate aldolase could be modified with *p*-mercuribenzoate without loss of enzymatic activity. Also, KOWAL *et al.*[21] have shown that the addition of fructose 1,6-diphosphate or phosphate protected 4–6 sulfhydryl groups of fructosediphosphate aldolase from reaction with *N*-ethylmaleimide or chlorodinitrobenzene and from inactivation by these reagents.

EXPERIMENTAL PROCEDURE

Materials

Fish muscle from the Antarctic fishes was collected in Antarctica during the summers (November–January) of four different years. *T. borchgrevinki* were hooked on lures, and were 6–12 inches long. *Dissostichus mawsoni* were captured alive from seals, and were 3–5 feet long. The fishes were maintained at approx. 0° before muscle samples were obtained by excision of lateral slices. The muscle was frozen immediately and taken back to the University of California, Davis. Rainbow trout *(Salmo gairdnerii)* were obtained from the American River Hatchery in Sacramento.

Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Inc., and CM-cellulose (CM-22) and DEAE-cellulose (DEAE-22) from Whatman Co. Fructose 1,6-diphosphate (sodium salt), fructose 1-phosphate (sodium salt), crystalline glyceraldehyde-3-phosphate dehydrogenase, and NAD^+ were purchased from Sigma

Chemical Co., and crystalline rabbit muscle fructosediphosphate aldolase from Boehringer Mannheim Corp. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich Chemical Co. All other reagents were of analytical grade.

Methods

Purification of fish fructosediphosphate aldolases. Attempts to crystallize fish fructosediphosphate aldolase by the method of TAYLOR *et al.*[12] were successful, but the specific activity of 3 times crystallized fructosediphosphate aldolase was low compared to that of rabbit muscle fructosediphosphate aldolase. Disc electrophoresis of the 3-times recrystallized preparations of fructosediphosphate aldolases revealed large amounts of impurities. A method of purification utilizing $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography, and fractionation by Sephadex G-200 was therefore developed. $(NH_4)_2SO_4$ fractionation resulted in precipitation of most of the fructosediphosphate aldolase activity from *T. borchgrevinki* and *D. mawsoni* muscle extracts at 40–45% saturation. However, since we were also studying lactic dehydrogenase in our laboratory, the first step in purification was precipitation at 60% saturation in $(NH_4)_2SO_4$ in order to precipitate the lactic dehydrogenase in the same initial step.

Dialysis experiments showed that *D. mawsoni* muscle fructosediphosphate aldolase was stable in a Tris–HCl buffer (pH 6.5) containing 1 mM β-mercaptoethylamine and 1 mM EDTA. Further purification of *D. mawsoni* fructosediphosphate aldolase was done by repeated column chromatography on CM-cellulose utilizing the preceding buffer system. As a final step the fructosediphosphate aldolase was fractionated on a Sephadex G-200 column and stored in a 60% saturated solution of $(NH_4)_2SO_4$ containing the buffer. Except for an initial chromatographic purification on a DEAE-cellulose column, the purification of *T. borchgrevinki* fructosediphosphate aldolase was done in essentially the same manner. However, dialysis experiments indicated that *T. borchgrevinki* muscle fructosediphosphate aldolase was stable in a Tris–HCl buffer (pH 6.7) containing 1 mM β-mercaptoethylamine, 1 mM EDTA, and 2% glycerol. The 2% glycerol was necessary for maintaining the stability of *T. borchgrevinki* fructosediphosphate aldolase.

Amino acid analysis. Except for tryptophan, amino acid analyses were done using a Technicon Autoanalyzer and a standard 21-h run. Duplicate samples were hydrolyzed in 6 M HCl at 110° for 22, 48, and 72 h (ref. 25) and values extrapolated to zero time. Tryptophan was determined spectrophotometrically by the method of GOODWIN AND MORTON[26].

Ultracentrifugal studies. Sedimentation velocity was determined with a Beckman model E analytical ultracentrifuge with schlieren optics. Determinations were made at 59 780 rev./min at 3 and 20°. Protein concentration varied from 0.15 to 0.30%. The buffer used was 0.10 M Tris–HCl (pH 6.8) containing 1 mM β-mercaptoethylamine and 1 mM EDTA. Molecular weights were determined by low-speed sedimentation equilibrium experiments[27]. Rayleigh interference optics was used. The fructosediphosphate aldolase concentration was 0.2% and the experiment was done at 3° and 6569 rev./min. The partial specific volume of 0.742 cm^3/g determined for rabbit muscle fructosediphosphate aldolase at 20° by TAYLOR AND LOWRY[28] was used. This value was corrected to 3° by subtracting 0.001 cm^3/g per degree[28].

Reaction of sulfhydryl groups with DTNB. Sulfhydryl groups were determined with DTNB[29]. Comparative determinations were done with and without the addition

of sodium dodecyl sulfate to differentiate between the more available and less available sulfhydryl groups. To 0.5 mg of fructosediphosphate aldolase in 3 ml of 0.01 M sodium phosphate buffer (pH 8.0), 0.02 ml of 0.01 M DTNB solution in 0.1 M sodium phosphate buffer (pH 7.0) were added. Reactions were done at 0, 25, and 45°. For the reaction in sodium dodecyl sulfate, 3 ml of 0.96% sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 8.0) were added. The progress of the reaction was followed by monitoring the increase in absorbancy at 412 mμ.

Fructosediphosphate aldolase assays. Fructosediphosphate aldolase activity was assayed by two methods. In the hydrazine assay system of JAGANNATHAN *et al.*[30], 2 ml of 3.5 mM hydrazine sulfate containing 0.1 M Tris–HCl and 1 mM EDTA (pH 6.8) were added to 1 ml of 12 mM fructose 1,6-diphosphate. Different amounts of fructosediphosphate aldolase were added and the change in absorbancy at 240 mμ was followed for 10 min.

A slight modification of the method introduced by WARBURG AND CHRISTIAN[31] was also used. A typical reaction mixture contained 0.1 ml of 12 mM fructose 1,6-diphosphate, 0.1 ml of 0.51 M $Na_2HAsO_4 \cdot H_2O$, 0.01 ml of 3 mM NAD^+, 2.5 ml of 0.2 M Tris–HCl at the desired pH, 0.1 ml of 0.6 M cysteine–HCl (pH 6.8), and 0.1 ml of 2 mg/ml glyceraldehyde-3-phosphate dehydrogenase. Fructose-1,6-diphosphate and the enzyme were added last. The reaction was followed by the change in absorbancy at 340 mμ.

All assays were done with a Cary Model 15 recording spectrophotometer. Temperature was regulated with a Neslab constant temperature bath to ± 0.05° at the different temperatures indicated.

RESULTS

Purification of fish muscle fructosediphosphate aldolase

Table I indicates the results obtained from the different steps in the purification of *D. mawsoni* muscle fructosediphosphate aldolase. A 17-fold purification of the initial extract was obtained. The final specific activity was slightly higher than that of the commercial rabbit muscle fructosediphosphate aldolase used. After electrophoresis on either acrylamide disc gel or starch gel, the purified fructosediphosphate

TABLE I

PURIFICATION OF FRUCTOSE DIPHOSPHATE ALDOLASE FROM 600 g OF *D. mawsoni* MUSCLE

All enzyme assays were done at 25° with the hydrazine coupling method. A unit is defined as a change in absorbance at 240 mμ of 1.00 per min. Specific activity equals $\Delta A_{240\ m\mu}$/min per mg protein per ml of reaction mixture.

Purification step	*Amount protein (mg)*	*Total units*	*Specific activity*	*Recovery (%)*
Crude extract	13 000	22 500	1.74	100
60% $(NH_4)_2SO_4$ ppt.	7 800	16 800	2.16	75
1st CM-cellulose chromatography	460	6 900	15.0	31
2nd CM-cellulose chromatography	240	4 500	18.8	20
3rd CM-cellulose chromatography	70	1 900	27.3	8
Sephadex G-200	60	1 800	30.0	8

aldolase appeared as one spot when stained with either aniline blue black or the substrate stain for fructosediphosphate aldolase[14]. *T. borchgrevinki* fructosediphosphate aldolase was purified about 18-fold, and its specific activity was approximately that of the commercial rabbit fructose diphosphate aldolase. The fish fructosediphosphate aldolase appeared homogeneous when electrophoresed and stained.

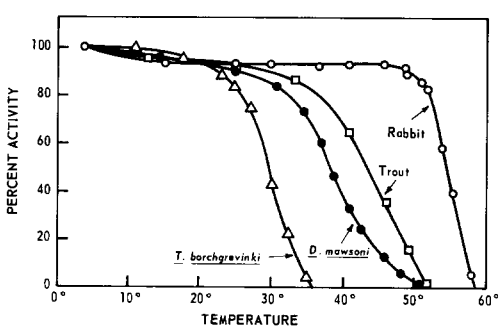
Temperature stabilities of purified fructosediphosphate aldolases

Comparative temperatures for half inactivation of the fructosediphosphate aldolases were determined by heating the enzymes in a solution of 0.2 M Tris–HCl, 2 mM β-mercaptoethylamine, and 1 mM EDTA (pH 6.8) for 30 min at the indicated temperatures (Fig. 1). Under these conditions the temperatures for half inactivation of *T. borchgrevinki*, *D. mawsoni*, and rabbit muscle fructosediphosphate aldolases were 29, 38, and 55°, respectively. Data on rainbow trout were obtained for muscle extracts, and were compared with data for extracts of *D. mawsoni* muscle. The temperature for half inactivation of the trout fructosediphosphate aldolase was 44°, while that of *D. mawsoni* was 38°, the same as for the purified enzyme.

Reactions with DTNB

The total numbers of sulfhydryl groups, as determined by reaction with DTNB in the presence of sodium dodecyl sulfate, were 16–17 for both of the fish enzymes and 28 for the rabbit enzyme.

When the different fructosediphosphate aldolases were reacted with DTNB at various temperatures, both the relative reactivities of the sulfhydryl groups and the relative rates of inactivation of the enzymatic activities were in the following order: *T. borchgrevinki*, *D. mawsoni*, and rabbit. At 0° and 3 h in the absence of sodium dodecyl sulfate, the *T. borchgrevinki* and *D. mawsoni* fructosediphosphate aldolase were inactivated nearly 90 and 25%, respectively, at which time there was no detectable inactivation of the rabbit enzyme (Fig. 2). With the fish enzymes, approx. 5



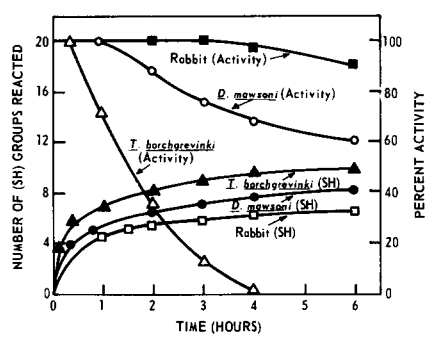


Fig. 1. Temperature stability of different fructosediphosphate aldolases. Incubations were done at the indicated temperatures for 30 min. The buffer used was 0.2 M Tris–HCl, 2 mM β-mercaptoethylamine, 1 mM EDTA (pH 6.8). Data were obtained with purified enzymes except with the trout enzyme, which was done with a muscle extract.

Fig. 2. Reactions of the sulfhydryl groups of different fructosediphosphate aldolases with DTNB at 0° and the concomitant losses of enzymatic activity. Concentrations of fructosediphosphate aldolase and DTNB were 0.167 mg/ml and 67 μM, respectively. Buffer was 0.01 M sodium phosphate (pH 8.0).

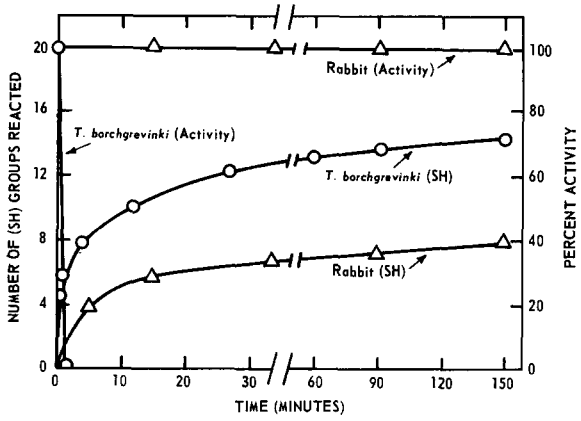


Fig. 3. Reactions of the sulfhydryl groups of different fructosediphosphate aldolases with DTNB at 25° and the concomitant loss of enzymatic activity. (Other conditions as for Fig. 2.)

or 6 sulfhydryls reacted before inactivation was initiated. At 25°, both of the fish enzymes were rapidly inactivated and their sulfhydryl groups reacted rapidly (Fig. 3). Nearly all of the sulfhydryls reacted after 3 h. Inactivation of the *T. borchgrevinki* fructosediphosphate aldolase occurred when approx. 6 sulfhydryls had reacted. In contrast, no detectable inactivation occurred when 6–7 of the rabbit sulfhydryls had reacted after a much longer period of reaction. However, rabbit muscle fructosediphosphate aldolase was completely inactivated with DTNB at 25° in the presence of sodium dodecyl sulfate or at 45° in the absence of sodium dodecyl sulfate.

Partial protection of the sulfhydryl groups of the fructosediphosphate aldolases of both fishes to DTNB at 25° was obtained by the presence of fructose 1,6-diphosphate. Under these conditions, no inactivation was noted until more than 6 sulfhydryls had reacted.

Specificity for substrate and inhibitors

The fructosediphosphate aldolases of *D. mawsoni*, *T. borchgrevinki*, and rabbit muscle were enzymatically inactive with fructose 6-phosphate, glucose 1-phosphate, or glucose 6-phosphate as substrates. Activity was found with fructose 1-phosphate as substrate and the ratio of activity with fructose 1,6-diphosphate to activity with fructose 1-phosphate was about 50 for all of the fructosediphosphate aldolases. All the fructosediphosphate aldolases were inactivated by heavy metal ions such as Ag^{+}, Cu^{2+}, and Mg^{2+}.

Amino acid compositions

The amino acid composition of *T. borchgrevinki* and *D. mawsoni* fructosediphosphate aldolases were very similar (Table II). The fish fructosediphosphate aldolases, however, differed significantly from rabbit muscle fructosediphosphate aldolase in having lower amounts of cysteine and higher amounts of methionine, valine, and phenylalanine. Also, they had about 8 more acidic groups and about 6 more basic groups. Total hydroxy amino acids were about the same in all three fructosediphosphate aldolases. Except for the difference in cysteine, the other amino acids which are usually considered necessary for the activity of rabbit muscle fructosediphosphate

TABLE II

AMINO ACID COMPOSITION OF FRUCTOSEDIPHOSPHATE ALDOLASES

Values are number of residues per 160 000 g.

Constituents	*Fructose diphosphate aldolases*		
	*Rabbit**	*T. borch-grevinki*	*D. mawsoni*
Aspartic acid	115	106	107
Threonine	82	91	94
Serine	79	66	67
Glutamic acid	152	171	167
Proline	88	80	79
Glycine	123	108	109
Alanine	165	161	164
Valine	61	91	85
Cysteine	28	16	15
Methionine	13	23	24
Isoleucine	57	65	70
Leucine	131	117	119
Tyrosine	41	46	47
Phenylalanine	27	45	46
Lysine	105	107	110
Histidine	43	44	44
Arginine	61	64	64
Tryptophan**	12	12	10

* Data taken from RIORDAN AND CHRISTEN[23].
** Determined spectrophotometrically.

aldolase—lysine, histidine, and tyrosine—were present in essentially equivalent amounts in the three fructosediphosphate aldolases. The value for cysteine agreed with that found by the DTNB reaction and the value found spectrophotometrically, for tyrosine agreed with that determined using the amino acid analyzer.

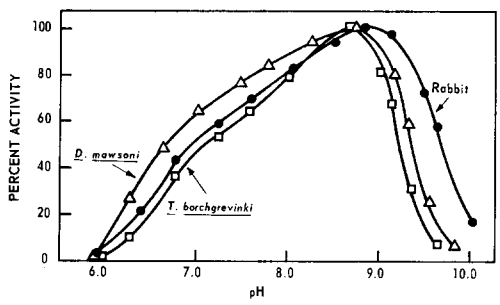
Ultracentrifugal analyses

Preparations of the Antarctic fish fructosediphosphate aldolases gave a single peak on velocity sedimentation in the analytical ultracentrifuge.

By using a $\bar{v}$ determined for rabbit muscle fructosediphosphate aldolase and correcting it to 3° the molecular weights of *T. borchgrevinki* and *D. mawsoni* fructosediphosphate aldolases were found to be 169 000 and 162 000, respectively. These values are in agreement with the value of 160 000 found for rabbit muscle fructosediphosphate aldolase[10], as the molecular weight determinations give values which are ± 5%. The $s^0_{20,w}$ values determined at 3° for *T. borchgrevinki* and *D. mawsoni* fructosediphosphate aldolases were 7.80 and 7.82 S, respectively. These agree with the values of 8.0 S by KAWAHARA AND TANFORD[10], 7.9 S by STELLWAGER AND SCHACHMAN[32], and 7.8 S by DEAL *et al.*[33] reported for rabbit muscle fructosediphosphate aldolase. A $s^0_{20,w}$ value of 7.70 S was found for *D. mawsoni* fructosediphosphate aldolase at 20°, but at 35° the sample aggregated.

Effect of pH on activity

The optimum pH is about 9.0 for all of the fructosediphosphate aldolases studied



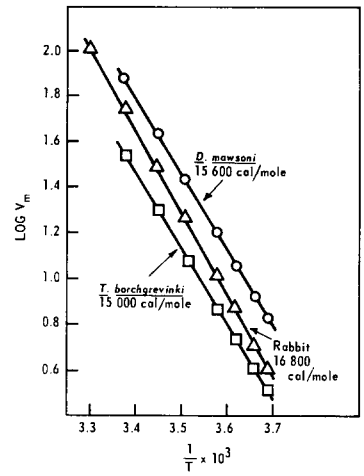


Fig. 4. Effect of pH on fructosediphosphate aldolase activities. pH was controlled with 0.2 M Tris–HCl.

Fig. 5. Activation energies of different fructosediphosphate aldolases in 0.2 M Tris–HCl, 1 mM β-mercaptoethylamine, 1 mM EDTA (pH 7.0).

(Fig. 4). The assay system used was the glyceraldehyde-3-phosphate dehydrogenase-coupled system of WARBURG AND CHRISTIAN[31]. The pH was controlled with 0.2 M Tris–HCl. The activities at the low and high pH values were checked by varying the concentration of glyceraldehyde-3-phosphate dehydrogenase.

Effect of temperature on the Michaelis constant

The Michaelis constants at different temperatures were determined with the hydrazine-coupled method since the glyceraldehyde-3-phosphate dehydrogenase-coupled method involved a time lag for NADH production at low temperatures. This led to curved lines which were difficult to interpret. The hydrazine-coupled method

TABLE III

EFFECT OF TEMPERATURE ON THE K_m FOR FRUCTOSE 1,6-DIPHOSPHATE BY FRUCTOSEDIPHOSPHATE ALDOLASES

The assays were done by the hydrazine-coupled method at pH 6.8. The concentrations of fructose 1,6-diphosphate were varied from 120 μM to 4 mM and the fructosediphosphate aldolase concentration was 3 μg/ml.

Temp.	$K_m \times 10^4$ *(M)*		
	Rabbit	*T. borch-grevinki*	*D. mawsoni*
−2°	1.8	1.66	1.83
0°	2.4	1.45	1.36
4°	4.9	2.1	0.835
8°	2.1	1.13	0.599
14°	0.9	0.89	0.555
21°	1.1	1.39	0.800
27.5°	2.1	1.50	0.890

gave straight lines, and the pH optimum for its reaction did not change in the temperature range studied. The concentration of fructose 1,6-diphosphate used ranged from 4 mM to 120 μM and the fructosediphosphate aldolase concentration was about 3 μg/ml. There were only small changes in K_m with changes in temperature, but a probable minimum K_m at about 14° was observed for all the fructose diphosphate aldolases under the conditions used (Table III).

Activation energy

The activation energies for the reaction catalyzed by fructosediphosphate aldolase were determined by plotting $1/T$ against log v_{max}, where T was the absolute temperature and v_{max} the maximum velocity. The values for rabbit, *D. mawsoni*, and *T. borchgrevinki* fructosediphosphate aldolases were 16 800, 15 600 and 15 000 cal/mole, respectively (Fig. 5). Since the activation energies are $\pm$ 2000 cal/mole, we could not conclude that there were significant differences in the activation energy of the reaction catalyzed by the different fructosediphosphate aldolases.

DISCUSSION

Two of the primary properties to consider in studying enzymes from cold-adapted species are the effects of temperature on the stabilities and on the activation energies of the enzymes. Heating experiments showed that the muscle fructosediphosphate aldolases from the Antarctic fishes were very heat labile compared to rabbit fructosediphosphate aldolase. The fructosediphosphate aldolase of the trout, a cool water fish, was also more labile than that of the rabbit, but more stable than that of *D. mawsoni*, the more stable of the two Antarctic ones. The fructosediphosphate aldolases of two comparatively warm water fishes, carp[34] and tuna[35], have been reported to be less stable than rabbit muscle fructosediphosphate aldolase.

Complete reaction of all the sulfhydryl groups of *D. mawsoni* and *T. borchgrevinki* fructosediphosphate aldolases with DTNB at 25° in the absence of sodium dodecyl sulfate was in agreement with the greater lability of the fish fructosediphosphate aldolases at 25°. At 25° only about a third of the sulfhydryl groups of rabbit muscle fructosediphosphate aldolase reacted with DTNB in the absence of sodium dodecyl sulfate, but at 45° all of the sulfhydryl groups reacted with DTNB in the absence of sodium dodecyl sulfate. Thus, both fish fructosediphosphate aldolases appear to be in a partially unfolded state at 25° and rabbit muscle fructosediphosphate aldolase does not reach that state until it is heated to about 45°. The reason for this lability is not known at this time, but it might be due to decreased intramolecular hydrogen bonding. However, although relative reactivities of side chains of amino acids can frequently be used as indications of protein conformation, loss of activity from chemical modification may be due to a secondary effect and do not necessarily prove essentiality of the groups modified. This is particularly the case for the reaction of DTNB with the sulfhydryl groups of proteins[29], because the products can be the disulfides formed from either two protein sulfhydryls or from a protein sulfhydryl and the aromatic mercaptan of the reagent[36,37]. These two products might have very different properties.

HOCHACHKA AND SOMERO[38] postulated that in poikiliotherms temperature effects on enzyme affinity for its substrate may be more important to the organism

than temperature effects on maximum velocity. They based their reasoning on the assumption that *in vivo* substrate levels may be too low for maximum enzyme activity. A minimum Michaelis constant (K_m) for lactate dehydrogenase was found at the environmental temperature of the fishes studied. The K_m value of pyruvate kinase for one of its two substrates, phosphoenolpyruvate, was also reported to be at a minimum at the habitat temperature of the fishes studied[39]. However, in the present studies when K_m values were determined for the different muscle fructosediphosphate aldolases a minimum was not found at the environmental temperature of the cold-adapted fishes, but possibly at about 14°. In the temperature range −2 to 27.5° rabbit muscle fructosediphosphate aldolase also had a slightly lower K_m for fructose 1,6-diphosphate at about 14°. GREENE[7] did not find a minimal K_m for glyceraldehyde-3-phosphate dehydrogenase from *D. mawsoni*. Thus for fructosediphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase no compensation in affinity for substrate seems to have occurred on cold adaptation.

There are two different lines of reasoning that might be used in considering the effect of cold adaptation on the activation energy of enzymatic reactions. One is that the activation energy should be high, for any increase in temperature will give reactions catalyzed by any given enzyme a larger increase in activity than reactions with lower activation energies. The other line of reasoning is that cold adaptation should decrease the activation energy and reduce the sensitivity of the organism to temperature change. This had been interpreted as occurring in several insects acclimated to colder temperatures[40]. Furthermore, in reactions with lower activation energies there should be a larger number of molecules with sufficient energy to react. This should result in a faster rate of reaction at any given temperature. This might be a more logical situation for cold-adapted Antarctic fishes in McMurdo Sound because the temperature is constant and cold at approx. −1.8°. GREENE[7] found that glyceraldehyde-3-phosphate dehydrogenase from *D. mawsoni* had an activation energy of 14 000 ± 1700 cal/mole and rabbit glyceraldehyde-3-phosphate dehydrogenase 18 150 ± 1500 cal/mole. Cod and lobster also have glyceraldehyde-3-phosphate dehydrogenase with activation energies of 14 500 cal/mole[41]. A similar difference in activation energy in the reaction catalyzed by the different fructosediphosphate aldolases has not been found. An average of three determinations gave a value of 16 800 cal/mole for rabbit muscle fructosediphosphate aldolase, 15 600 cal/mole for *D. mawsoni* fructosediphosphate aldolase, and 15 000 cal/mole for *T. borchgrevinki* fructosediphosphate aldolase.

ACKNOWLEDGMENTS

The authors gratefully appreciate some of the initial observations by Dr. Herman T. Miller who accompanied one of the research teams in Antarctica and the general assistance of Dr. Richard G. Allison and David T. Osuga who also accompanied one of the research teams in Antarctica.

This work was supported in part by Grant GA-3919 from the Division of Environmental Science, National Science Foundation. Parts of this material were presented at the 158th meeting of the American Chemical Society, Sept. 8–12, 1969, New York, N.Y.

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