BBA 65704

PURIFICATION AND CHARACTERIZATION OF THE FRUCTOSE DIPHOSPHATE ALDOLASES FROM *ANACYSTIS IS NIDULANS* AND *SAPROSPIRA THERMALIS*

JAMES M. WILLARD* AND MARTIN GIBBS

Department of Biochemistry, Cornell University, Ithaca, N.Y. and Department of Biology, Brandeis University, Waltham, Mass. (U.S.A.)

(Received October 27th, 1967)

SUMMARY

1. Fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) has been purified 970-fold from the blue-green alga, *Anacystis nidulans*, by a procedure involving $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption and DEAE-cellulose chromatography. The purified enzyme has a sharp pH optimum at 7.6 for Fru-1,6-P_2 cleavage and an apparent molecular weight of 137 000. For catalytic activity the aldolase exhibits an absolute requirement for both a divalent metal and a thiol acid. The Michaelis constant for Fru-1,6-P_2 is 0.22 mM; for sedoheptulose-1,7-P_2 is 10 mM; and for Fe^{2+} is 3.8 μM. The maximal velocity at 26° for Fru-1,6-P_2 is 5200.

2. The aldolase from the Flexibacterium, *Saprospira thermalis*, has been purified 240-fold by a procedure involving ethanol and $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. The purified enzyme has a sharp pH optimum at 8.0 for Fru-1,6-P_2 cleavage and an apparent molecular weight of 137 000. The aldolase requires cysteine, Zn^{2+} and K^+ for maximum activity. The Michaelis constant and maximal velocity for Fru-1,6-P_2 is 0.19 mM and 6600, respectively.

3. The properties of these metal-requiring aldolases are almost identical to the purified yeast enzyme. Furthermore, since it appears that bacteria possess only metal-dependent aldolases, the occurrence of such an aldolase in Anacystis reinforces the argument that the blue-greens represent a common link between the bacteria and higher plants.

INTRODUCTION

Fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) is an essential component of the classical glycolytic, fermentative and photosynthetic carbon reduction pathways[1]. Although the mamma-

* Present address: Department of Biochemistry, CASE Western Reserve University, Cleveland, Ohio, U.S.A.

lian[2,3], yeast[3] and higher plant[4,5] aldolases have been well characterized, little attention has been focused on the aldolases from the bacteria and photosynthetic organisms.

Morphologically, bacteria and blue-green algae are composed of prokaryotic cells, *i.e.* cells possessing no distinct intracellular organelles within their cytoplasm. This contrasts with all other organisms containing eukaryotic or nucleated cells. Since the blue-green algae carry out photosynthesis, it has been suggested that they may represent a common link between bacteria and higher plants. To date all bacterial aldolases detected in crude extracts appear to be metal-dependent or Type II aldolases according to the classification of RUTTER[6]. Preliminary support for this common link by the blue-green algae was afforded by the recent demonstration of metal activated aldolase activity in crude extracts of *Anacystis nidulans*[6-8] and the photosynthetic bacterium, *Rhodopseudomonas spheroides*[7]. In the present communication we present the purification and characterization of a metal-requiring aldolase from *A. nidulans*. A partial purification and characterization of the apparent metal-requiring aldolase of *Saprospira thermalis*, considered to be a 'colorless' blue-green form, was also made to ascertain if its aldolase has properties similar to that of the blue-green enzyme. If the aldolases prove similar, the argument that this alga represents a colorless blue-green form would be reinforced[9].

METHODS

Organisms

The blue-green alga, *A. nidulans* was obtained from Dr. J. MYERS, the University of Texas, Austin. Ten 1-l batches of cells were cultured photoautotrophically at 30° with 1% CO_2–99% air on Medium C of KRATZ AND MYERS[10]. Cells were grown under continuous fluorescent illumination of 500-ft candles and harvested by centrifugation after 5 days of growth.

The Flexibacterium, *S. thermalis*, was obtained from Dr. R. A. LEWIN, Scripps Institution of Oceanography, University of California, La Jolla. 5-l cultures were grown aerobically at 25° as previously described[9] and harvested after 4 days of growth.

Preparation of cell-free extracts

1–2 g wet weight Anacystis and 10 g Saprospira cells were sonicated in 0.05 M Tris–HCl (pH 7.6) at full power by a Branson Model S-75 Sonifier. To facilitate greater yields in aldolase units from Anacystis, the cell suspension was deaerated 4 min with N_2 just prior to sonication. Following centrifugation at 10 000 × *g* the supernatant solutions constituted cell-free extracts.

Determination of aldolase activity

Two methods of aldolase assay were employed and yielded comparable results: (A) the colorimetric method of SIBLEY AND LEHNINGER[11] and (B) the spectrophotometric method of WU AND RACKER[12]. All rates were linear with time and stoichiometric with enzyme.

The colorimetric assay was performed at 37° in 2.5-ml reaction mixtures consisting of 40 mM Tris–HCl (pH 7.6), 56 mM hydrazinesulfate (pH 7.5), 4 mM Fru-1,6-P_2 aldolase and other additions as desired. In all cases Fru-1,6-P_2 was added last. Reactions were terminated after 20 min by the addition of 2.0 ml cold 10% trichloro-

acetic acid. Blanks consisted of adding Fru-1,6-P_2 after the addition of trichloroacetic acid. Controls with no aldolase present were run when cofactors or inhibitors were employed. After development of the triose chromagens[11] the resultant absorption at 540 nm was determined in 1-cm cuvettes with a Beckmann DU spectrophotometer.

Spectrophotometric assays were performed at either 26° or 37° employing the coupling system of triose phosphate isomerase and α-glycerol phosphate dehydrogenase. In a final volume of 1.0 ml the reaction mixture consisted of 40 mM Tris–HCl (pH 7.6), 6 μg crystalline coupling enzymes, 0.2 mM DPNH, 5 mM Fru-1,6-P_2, aldolase and other additions as required. Reference cuvettes contained no DPNH and controls lacked Fru-1,6-P_2 or aldolase. Depending on the experiment, aldolase or Fru-1,6-P_2 was added last. DPNH oxidation at 340 nm was followed in 1-cm cuvettes with a Beckmann DU spectrophotometer equipped with a Gilford Model 2000 multiple absorbance recorder. Temperature was controlled by use of a Haake Model F circulator. Fe^{2+} refers to $Fe(NH_4)_2(SO_4)_2$.

Definition of aldolase unit

A unit of aldolase activity is defined as the amount of enzyme catalyzing the cleavage of 1 μmole Fru-1,6-P_2 per h at a specified temperature of 26° or 37°. The specific activity is given as units/mg protein. Protein was determined by the method of LOWRY *et al.*[13] using crystalline bovine serum albumin as standard, and spectrophotometrically as described by WARBURG AND CHRISTIAN[14].

DEAE-cellulose chromatography

50-g lots of DEAE-cellulose (Selectacel preparations from Brown Co.) were suspended in 2 l 0.1 M NaOH and 0.1 M NaCl and stirred for 10 min. The resin was collected on a Buchner funnel and once washed as before. After washing the resin with H_2O to about pH 8 it was resuspended in a solution containing 0.1 M HCl and 0.1 M NaCl, filtered and washed with H_2O to pH 5. The resin was tored in distilled H_2O until used. Columns of desired length were packed under gravity.

Apparent molecular weights

Molecular weights were determined by the sucrose gradient method of MARTIN AND AMES[15]. Sucrose gradients of 5 to 25% in 0.05 M Tris–HCl (pH 7.6) were employed. Catalase and lactic acid dehydrogenase (a gift of Dr. N. O. KAPLAN) served as internal markers. Following centrifugation, 3-drop fractions were analyzed.

pH Profiles of aldolases

The buffers and the pH at which each was employed are: (1) histidine, 6.0 and 6.3; (2) imidazole, 6.7, 7.0 and 7.3; (3) glycylglycine, 7.6, 8.0 and 8.5; and (4) glycine, 9.0. After calculating the amount of salt present at each pH, that amount of buffer yielding a final ionic strength of 0.05 was employed.

RESULTS

Purification of Anacystis aldolase

A summary of the purification procedure for the Anacystis aldolase is shown in

Table I. The final preparation had a specific activity of 7680 representing a 970-fold purification.

Freshly prepared cell-free extract (29 ml) was brought to 35% satn. with 7.2 g solid $(NH_4)_2SO_4$ and centrifuged at 10 000 × *g*. The resultant pellet was discarded and the supernatant solution brought to 75% satn. with a further 8.2 g $(NH_4)_2SO_4$. Following centrifugation the 75% supernatant was discarded and the pellet was suspended in 4 ml deaerated (by N_2) 0.05 M Tris–HCl (pH 7.6). This 35–75% $(NH_4)_2SO_4$ fraction was stable for several months when stored under N_2 at −15°.

61 ml calcium phosphate gel suspension in pH 7.6 0.05 M Tris–HCl (containing

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR *Anacystis nidulans* ALDOLASE

Aldolase activity was determined by the colorimetric assay method at 37° with 8 mM cysteine and 1 mM Fe^{2+} present.

Fractionation step	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery*
Cell-free extracts: sum of 3 lots	3876	8	100
35–75% $(NH_4)_2SO_4$: sum of 3 lots	2300	35	59
Stored and pooled 35–75% $(NH_4)_2SO_4$	2315	35	60
Calcium phosphate gel and 35–80% $(NH_4)_2SO_4$ eluate	1995	125	51
Combined Sephadex G-25 fractions	1885	136	48
Combined DEAE-cellulose fractions	1182	—	30
80% $(NH_4)_2SO_4$ fraction off DEAE-cellulose	645	7680	17

16.5 mg gel dry wt./ml) was added to 10 ml of three pooled 35–75% $(NH_4)_2SO_4$ fractions (66.25 mg protein). After 10 min magnetic mixing the suspension was centrifuged and the supernatant solution was discarded. The calcium phosphate gel pellets were extracted twice with 30 ml each 35% satd. $(NH_4)_2SO_4$. The 35% $(NH_4)_2SO_4$ solution was raised to 80% satn. with 13.6 g solid $(NH_4)_2SO_4$ and centrifuged. The resultant precipitate was suspended in 4.5 ml 0.05 M Tris–HCl (pH 7.6) and recentrifuged.

The 35–80% $(NH_4)_2SO_4$ fraction (5.3 ml) was passed through a 1.5 cm × 15 cm Sephadex G-25 column previously equilibrated with 0.05 M Tris (pH 7.6). A fraction of 2.3 ml was collected at 0.8 ml/min. Fractions 6 through 11 were pooled and 13.5 ml applied directly to a 1.5 cm × 23 cm DEAE-cellulose column equilibrated with 0.05 M Tris–HCl (pH 7.6). A fraction of 4.6 ml was then collected at 1.9 ml/min. Stepwise gradient elution with NaCl solutions of 0.05, 0.1, 0.15, 0.2 and 0.35 M concentrations was performed and aldolase and phycocyanin (reflected by its absorption at 615 nm) were located (Fig. 1). The aldolase in Fractions 43 through 57 (67 ml) was concentrated by adding 37.8 g solid $(NH_4)_2SO_4$ to yield 80% satn. The resultant precipitate after centrifugation was suspended in a final 3.0 ml 0.05 M Tris–HCl (pH 7.6). This preparation was stable for several weeks when stored at −15° under air.

The purified enzyme did not contain DPN- or TPN-linked glyceraldehyde-3-*P* dehydrogenase activities, and, on a unit of activity basis, contained about 0.1% triose phosphate isomerase.

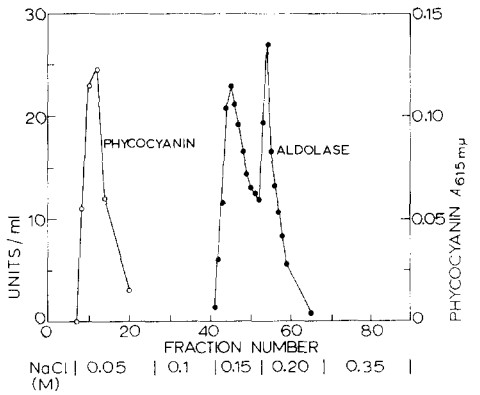


Fig. 1. DEAE-cellulose chromatography of Anacystis aldolase. Aldolase was assayed by the colorimetric method with a 10-min preincubation in 8 mM cysteine and 1 mM Fe^{2+}. The elution profile is described in the purification procedure.

Purification of Saprospira aldolase

A summary of the purification of the Saprospira aldolase is shown in Table II. The final preparation had a specific activity of 5100 representing a 244-fold purification.

To 147 ml of freshly prepared cell-free extract was added drop-wise 163 ml 95% ethanol (−10°) to yield 50% in ethanol. The suspension was centrifuged at −10°. The precipitate was aspirated 1 min then suspended in 40 ml 0.05 M Tris–HCl (pH 7.6) and recentrifuged. Forty ml of the 0–50% ethanol fraction was brought to 50% satn. with 40 ml satd. $(NH_4)_2SO_4$ and centrifuged. The supernatant solution was then

TABLE II

SUMMARY OF PURIFICATION PROCEDURE FOR *Saprospira thermalis* ALDOLASE

Aldolase activity was determined by the colorimetric assay method at 37° with 8 mM cysteine, 1 mM Zn^{2+}, and 0.1 M K^+ present.

Fractionation step	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*
Cell-free extracts: sum of 2 lots	11 550	21	100
0–50% ethanol fractions: sum of 2 lots	7 720	116	67
50–75% $(NH_4)_2SO_4$ fractions: sum of 2 lots	6 260	191	54
Stored and pooled 50–75% $(NH_4)_2SO_4$	5 900	195	51
Combined Sephadex G-25 fractions	5 000	157	43
Pooled DEAE-cellulose fractions:			
10–15	1 300	—	11
51–58	546	—	5
59–63	1 380	—	12
64–68	418	—	4
69–75	266	—	2
75% $(NH_4)_2SO_4$ fraction off DEAE-cellulose	908	5100	8

brought to 75% satn. with an additional 80 ml $(NH_4)_2SO_4$. Following centrifugation the precipitate was suspended in a final 9 ml 0.05 M Tris–HCl (pH 7.6).

Two pooled 50–75% $(NH_4)_2SO_4$ fractions (14 ml) were passed over a 2 cm × 15 cm Sephadex G-25 column equilibrated with 1 mM sodium phosphate (pH 6.8). The sample was washed through with 200 ml of the phosphate buffer. A 7.4-ml fraction was collected. Fractions 3 through 8 were pooled and 41 ml placed directly on a 1.5 cm × 23 cm DEAE-cellulose column equilibrated with 1 mM sodium phosphate (pH 6.8). Fractions of 5.5 ml were collected at 2.5 ml/min. Stepwise gradient elution with 0.01, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 M sodium phosphate (pH 6.8) was performed and aldolase was located by assay of the 280-nm peaks (Fig. 2). The aldolase in Fractions 51

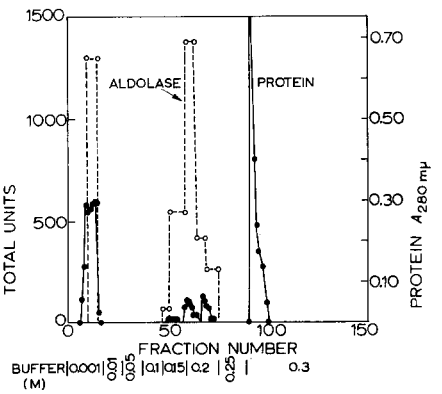


Fig. 2. DEAE-cellulose chromatography of Saprospira aldolase. Aldolase was assayed by the colorimetric method with a 10 min preincubation with 8 mM cysteine, 1 mM Zn^{2+} and 0.1 M K^+. The elution profile is described in the purification procedure.

through 68 (90 ml) was concentrated by adding 47.7 g solid $(NH_4)_2SO_4$ to yield 75% satn. The precipitate was suspended in 3.5 ml 0.05 M Tris–HCl (pH 7.6) during which time a large insoluble precipitate remained. After centrifugation the aldolase in the final supernatant solution (4.0 ml) was found to be quite stable when stored several weeks at −15°.

Similar to the Anacystis aldolase this preparation contained no detectable glyceraldehyde-3-*P* activity and, on a unity of activity basis, had about 0.1% triose phosphate isomerase.

Cofactor requirements of purified algal aldolases

The results obtained when the purified enzymes were assayed spectrophotometrically in the presence of various cofactors are given in Table III. The Anacystis aldolase was active only with both cysteine and Fe^{2+} (Zn^{2+} could not substitute for Fe^{2+}). K^+ had no effect upon the rate of cleavage. The Saprospira enzyme also had an absolute requirement for cysteine, but the enzyme was stimulated by Zn^{2+} and not by Fe^{2+}. Highest rates were always obtained when K^+ was present.

The Anacystis aldolase required about 10-min preincubation with cysteine and Fe^{2+} for maximal activity; while that of Saprospira required about 6-min preincubation with cysteine, Zn^{2+} and K^+. Optimal concentration of cofactors for the Anacystis enzyme were found to be 8 mM cysteine and 0.1 mM Fe^{2+}.

TABLE III

COFACTOR REQUIREMENTS OF PURIFIED ALGAL ALDOLASES

Aldolase units were determined by the spectrophotometric assay method. *A. nidulans* rates were determined at 37° after a 10-min preincubation; *S. thermalis* rates were determined at 26° with a 10-min preincubation.

Type aldolase	*Cofactor present*	*Units/ml*
A. nidulans	None	0
	8 mM cysteine	0
	1 mM Fe^{2+}	0
	8 mM cysteine + 0.1 M K^+	0
	8 mM cysteine + 1 mM Fe^{2+}	226
	8 mM cysteine + 0.5 mM Fe^{2+}	212
	8 mM cysteine + 1 mM Fe^{2+} + 0.1 M K^+	236
	8 mM cysteine + 1 mM Zn^{2+} + 0.1 M K^+	14
S. thermalis	None	0
	8 mM cysteine	63
	0.5 mM Zn^{2+}	10
	8 mM cysteine + 0.5 mM Fe^{2+}	53
	8 mM cysteine + 0.5 mM Zn^{2+}	96
	8 mM cysteine + 0.5 mM Zn^{2+} + 0.1 M K^+	174

TABLE IV

REPLACEMENT OF Fe^{2+} AND CYSTEINE BY OTHER METALS AND REDUCING AGENTS IN THE ASSAY OF *A. nidulans* ALDOLASE

Aldolase activity was determined at 37° by the spectrophotometric assay method. In Expt. I activity was determined with a constant 8 mM cysteine and 0.5 mM metal salts. In Expt. II 0.5 mM Fe^{2+} was present.

Expt. No.	*Cofactor present*	*% activation with cysteine Fe^{2+} = 100%*
I	$Fe(NH_4)_2(SO_4)_2$ = Fe^{2+}	100
	$FeSO_4$	94
	$FeCl_2$	87
	$Fe_2(SO_4)_3$	100
	$FeCl_3$	85
	$MnSO_4$	72
	$MnCl_2$	77
	$MgSO_4$	25
	$MgCl_2$	28
	$ZnSO_4$	25
	$ZnCl_2$	25
	$CuSO_4$	13
	$NiSO_4$	34
II	8 mM mercaptoethanol	0
	0.8 mM BAL	0
	8 mM GSH	20
	8 mM reduced thioglycollate	58

Effect of other divalent metals and reducing agents on the Anacystis aldolase

The Anacystis aldolase was assayed with a variety of other metals and reducing agents (Table IV). In the presence of cysteine, Fe^{3+} and Mn^{2+} salts, as well as other Fe^{2+} salts, could effectively replace $Fe(NH_4)_2(SO_4)_2$. Co^{2+} salts, under these conditions, interfered with the assay method. In the presence of Fe^{2+}, GSH and thioglycollate yield rates 20 and 58%, respectively, of those obtained with cysteine. β-Mercaptoethanol and BAL were uneffective. Reduced lipoate interfered with the assay method.

Evidence for metal in Anacystis aldolase

The small activation obtained when cysteine and Mg^{2+} were employed (Table IV) seemed to exclude the requirement that the metal undergo an oxidation–reduction during the enzymic cleavage of Fru-1,6-P_2. When a 5-fold excess of aldolase was employed for assay, cysteine alone was found to be active, yielding rates about 3% those obtained with both cysteine and Fe^{2+} present (Table V). Mg^{2+} salts had no effect upon

TABLE V

EVIDENCE FOR ENDOGENOUS METAL IN PURIFIED ANACYSTIS ALDOLASE

Expt. I employs the Anacystis aldolase in 5-fold excess to that amount employed in Table IV; Expt. II with the rabbit muscle enzyme. The spectrophotometric assay method was employed at 37°. 8 mM cysteine was present in all trials.

Expt. No.	*Assay condition*	*Units/ml*	*Type effect*
I	No inhibitor	7.7	—
	o-Phenanthroline		
	0.1 mM	0	100% inhibition
	0.05 mM	0	100% inhibition
	0.01 mM	2.9	63% inhibition
	2,2′-Bipyridine		
	0.1 mM	0	100% inhibition
	0.05 mM	1.4	81% inhibition
	0.01 mM	8.6	11% activation
II	No inhibition	53.0	—
	o-Phenanthroline, 0.1 mM	50.0	None
	2,2′-Bipyridine, 0.1 mM	55.0	None

this rate. Furthermore, the metal chelators, *o*-phenanthroline and 2,2′-bipyridine effectively inhibited the rates obtained with cysteine alone. The purified enzyme would appear to contain a metal, possibly iron. Although not presented, the same concentrations of chelators effectively inhibited when added after Fru-1,6-P_2, suggesting that cysteine, and possibly the metal are required for both activation and activity.

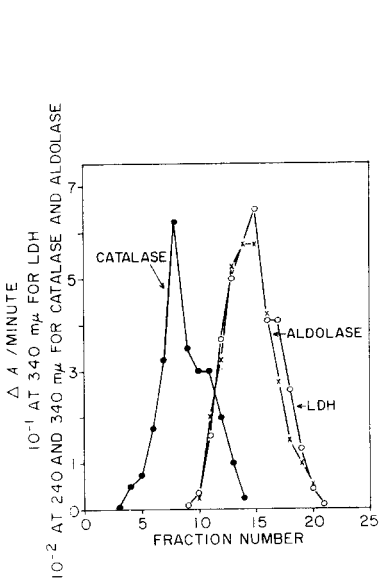
Apparent molecular weights

When determined by the sucrose density gradient method, the purified Anacystis aldolase was found to accompany lactic acid dehydrogenase (Fig. 3). Similar results were obtained with Saprospira aldolase. The molecular weights of both aldolases are, therefore, about 137 000 (ref. 21). These molecular weights were obtained with samples which were catalytically inactive. When the aldolases were first activated,

then centrifuged on gradients containing appropriate cofactors, the results obtained were identical to those obtained with the inactive species.

pH optima for Fru-1,6-P_2 cleavage

The Anacystis enzyme exhibited an optimum at pH 7.6 when determined at 26 and 37° (Fig. 4). The Saprospira enzyme had a similar optimum at pH 8.0. No difference was observed between Condition I (activating and assaying at the same pH) and Condition II (activating at pH 7.6, then assaying at varying pH).



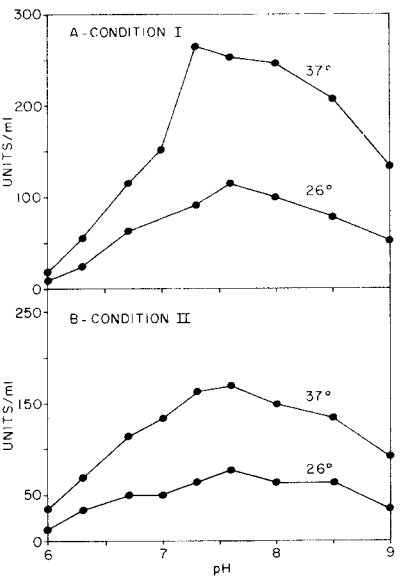


Fig. 3. Apparent molecular weight of Anacystis aldolase determined by the sucrose-gradient method. Sedimentation was run for 12 h at 35 500 rev./min at a final 18° on 5 to 25% sucrose gradient. Aldolase was determined by the spectrophotometric assay method at 37° with a 10-min preincubation with 8 mM cysteine and 0.1 mM Fe^{2+}. Lactic acid dehydrogenase (LDH) was determined spectrophotometrically at 26° as described by PESCE *et al.*[21]. Catalase was followed by measuring the extinction of H_2O_2 at 240 nm.

Fig. 4. pH profile of Anacystis aldolase. The spectrophotometric assay method at 26° and 37° was employed with a 10-min preincubation with 8 mM cysteine and 0.5 mM Fe^{2+}. Condition I is activating and assaying at the appropriate pH of the profile; Condition II is activating at pH 7.6, then assaying at the pH of the profile.

Substrate specificity of Anacystis aldolase

The Anacystis enzyme cleaved 4 mM sedoheptulose-1,7-P_2 at a rate 59% of that obtained with Fru-1,6-P_2. No activity was noted with Fru-1-P_1, ribulose-1,5-P_2, 2-keto,3-deoxyphosphogluconate, sorbose-1-P_1, sorbose-1,6-P_2, rhamnulose-1-P_1, or fuculose-1-P_1, each at 4 mM concentration.

Apparent kinetic constants of algal aldolases

The calculated kinetic constants for Fru-1,6-P_2, sedoheptulose-1,7-P_2 and Fe^{2+} for both aldolases have been calculated from LINEWEAVER–BURK[16] plots and are

TABLE VI

SUMMARY OF THE APPARENT KINETIC CONSTANTS OF THE PURIFIED ALGAL ALDOLASES

The spectrophotometric assay method was used throughout. The constants for Fru-1,6-P_2 were determined with a 10-min preincubation with 8 mM cysteine and 0.5 mM Fe^{2+} in the case of Anacystis; with 8 mM cysteine, 0.5 mM Zn^{2+} and 0.1 M K^+ in the case of Saprospira. The constants for sedoheptulosediphosphate were determined with 8 mM cysteine and 0.5 mM Fe^{2+}, and for Fe^{2+} with 8 mM cysteine. The v_{max} are expressed as moles Fru-1,6-P_2 cleaved per min per mole aldolase. The data for the yeast aldolase are taken directly from ref. 3.

Substrate or cofactor	*Temp. (°C)*	*A. nidulans*		*S. thermalis*		*Yeast*	
		K_m *(mM)*	v_{max}	K_m *(mM)*	v_{max}	K_m *(mM)*	v_{max}
Fru-1,6-P_2	26	0.16	5 200	0.19	6600	0.37	6900
	37	0.22	12 450	0.19	9550	—	—
Fe^{2+}	37	0.0038	—	—	—	—	—
Sed-1,7-P_2	37	10.0	—	—	—	—	—

summarized in Table VI. The apparent affinities of both aldolases for Fru-1,6-P_2, when determined in the presence of their respective cofactors, were identical. The Anacystis aldolase possesses a 57-fold higher affinity for Fe^{2+} than Fru-1,6-P_2 and appears to bind Fru-1,6-P_2 45-fold tighter than sedoheptulose-1,7-P_2.

DISCUSSION

The purification procedures for the Anacystis and Saprospira aldolases result in highly pure and stable preparations. The Anacystis enzyme contained no trace of the molecularly similar biliprotein, phycocyanin, which constitutes about 40% of the total soluble protein and has an isoelectric point of 4.5–5.0 and a minimal molecular weight of 138 000 (ref. 17).

For maximal activity, the Anacystis enzyme requires both cysteine and Fe^{2+}, and unlike the yeast enzyme is not stimulated by K^+. The Saprospira enzyme has cofactor requirements identical to those of the yeast enzyme, *i.e.* maximal activity results only with cysteine, Zn^{2+}, and K^+ present[3,6].

Both purified algal aldolases possess certain molecular and catalytic properties similar to the yeast enzyme. Both exhibit sharp pH optima for Fru-1,6-P_2 cleavage; and although having molecular weights twice that of the yeast enzyme, the algal aldolases have kinetic constants for Fru-1,6-P_2 which are identical to those of the yeast enzyme. It appears that the Type II, metal-requiring aldolases can be characterized as having 10-fold lower affinities for Fru-1,6-P_2 than Type I (non-metal requiring) aldolases, but with turnover numbers twice those of the Type I aldolases.

Unlike the Type I aldolases of mammalian liver and muscle, but similar to other well-characterized Type II aldolases (yeast, *Candida utilis*, *Aspergillus niger*), the Anacystis aldolase exhibited no activity with Fru-1-P_1. Of a variety of other sugar phosphates tried, only sedoheptulose-1,7-P_2 was cleaved. The observation that the Anacystis aldolase requires a finite incubation with cofactors before addition of Fru-1,6-P_2 to obtain activity, coupled with the much higher affinity for Fe^{2+} than Fru-

1,6-P_2, suggests Fe^{2+} is functioning catalytically, and the true substrate is Fru-1,6-P_2 and not a metal–substrate complex.

This investigation demonstrates unequivocally the presence of Type II, metal-requiring aldolases in blue-green algae and the morphologically related non-photosynthetic flexibacteria. Since the bacteria appear to possess only metal-dependent aldolases, the occurrence of a Type II aldolase in a photosynthetic prokaryotic cell, such as Anacystis, greatly reinforces the argument that the blue-greens may represent a common link between bacteria and higher plants. RUSSELL AND GIBBS[18] have described the preparation of highly purified aldolase from the autotrophically grown green alga, *Chlamydomonas mundana*, with certain properties similar to the Type I, muscle enzyme. Since no evidence for a Type I aldolase was detected in crude extracts of Anacystis[7] (*i.e.* without cofactors present no aldolase activity was detected) it would appear that a Type II enzyme can be associated with the photosynthetic carbon reduction cycle. However, when it is noted that other photosynthetic organisms, such as Euglena[6,19] and Chlamydomonas[18,20] appear to form Type II aldolases only when grown heterotropically on exogenous carbon sources, the uniqueness of the blue-green Type II aldolase functioning in its photosynthetic cycle becomes apparent. An assessment of the role of this aldolase in the blue-green algae must await a separation of the organelles carrying out the various aspects of their carbohydrate metabolism.

ACKNOWLEDGEMENTS

This investigation was supported by a Public Health Service predoctoral fellowship from the National Institute of General Medical Sciences to J. M. W. and by a grant of the National Science Foundation.

REFERENCES

1 M. GIBBS AND J. F. TURNER, *Modern Methods of Plant Analysis*, Vol. 3, Springer, Berlin, 1964, p. 519.
2 J. F. TAYLOR, A. A. GREEN AND G. T. CORI, *J. Biol. Chem.*, 173 (1948) 591.
3 O. C. RICHARDS AND W. J. RUTTER, *J. Biol. Chem.*, 236 (1961) 3177.
4 K. BROOKS AND R. S. CRIDDLE, *Arch. Biochem. Biophys.*, 117 (1966) 650.
5 R. FLURI, T. RAMA SARMA, AND B. L. HORECKER, *European J. Biochem.*, 1 (1967) 117.
6 W. J. RUTTER, *Federation Proc.*, 23 (1964) 1248.
7 J. M. WILLARD, M. SCHULMAN AND M. GIBBS, *Nature*, 206 (1965) 195.
8 C. VAN BAALEN, *Nature*, 206 (1965) 193.
9 R. A. LEWIN, *Can. J. Microbiol.*, 11 (1965) 135.
10 W. A. KRATZ AND J. MYERS, *Am. J. Botany*, 42 (1955) 282.
11 J. A. SIBLEY AND A. L. LEHNINGER, *J. Biol. Chem.*, 177 (1949) 859.
12 R. WU AND E. RACKER, *J. Biol. Chem.*, 234 (1959) 1029.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
15 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
16 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
17 C. O'HEOCHA, *Physiology and Biochemistry of Algae*, Academic Press, New York, 1962, p. 421.
18 G. K. RUSSELL AND M. GIBBS, *Biochim. Biophys. Acta*, 132 (1967) 145.
19 J. M. WILLARD AND M. GIBBS, *Plant Physiol.*, in the press.
20 G. K. RUSSELL AND M. GIBBS, *Plant Physiol.*, 41 (1966) 885.
21 A. PESCE, R. H. MCKAY, F. STOLZENBACH, R. D. CHAN AND N. O. KAPLAN, *J. Biol. Chem.*, 239 (1964) 1753.