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Nicotinamide Adenine Dinucleotide-specific Glyceraldehyde 3-Phosphate Dehydrogenase from *Pisum sativum*

PURIFICATION AND CHARACTERIZATION*

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SUMMARY

NAD$^+$-specific glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was purified from pea seeds to a specific activity in excess of 200 units per mg of protein, with a recovery of one-third of the initial activity. The specific activity is similar to that of the pure enzyme from other sources. On the basis of sedimentation velocity and sodium dodecyl sulfate gel electrophoresis studies, the enzyme appears to be homogenous. It is a tetramer, with a subunit molecular weight of approximately 36,000 to 37,000. Each subunit has the capacity to bind 1 mole of NAD$^+$, and the enzyme, as isolated, contains 2 to 3 moles of NAD$^+$ per tetramer. The amino acid composition is similar to that of the enzyme from other sources. The enzyme has a pH optimum between 8.5 and 9.0. Each of the substrates, but particularly glyceraldehyde 3-phosphate, is inhibitory at high concentrations.

In all major respects, the pea seed enzyme does not appear to differ markedly from glyceraldehyde 3-phosphate dehydrogenase from other sources.

NAD$^+$-specific glyceraldehyde 3-phosphate dehydrogenases (EC 1.2.1.12) have been purified from a variety of sources, and all are essentially identical. The enzyme is a tetramer with a molecular weight of 144,000 (1, 2). Each subunit can bind 1 mole of NAD$^+$ (3-5), and the enzyme from many sources is isolated with bound NAD$^+$ (6). The specific activity (6) and activity with NAD$^+$ analogs (7, 8) are similar for all sources. The enzyme from various sources does not differ markedly with respect to its sedimentation coefficient (6) or its amino acid composition (2, 6, 9), and for two sources, pig and lobster, there are extensive homologies in amino acid sequence (2).

There has been very little work done on the enzyme from higher plants. Hageman and Arnon (10) first reported the purification of the enzyme from pea seeds, and Schulman and Gibbs (11) also purified the enzyme from this source. In both cases, the specific activity was low (approximately 10 units[1] per mg of protein) and it is doubtful if these preparations were pure. It was therefore decided to purify the pea seed enzyme in order to determine if it is similar to the enzyme from other sources. In the following two papers, the steady state kinetics of the pea seed enzyme and studies on a partial reaction are reported. On the basis of these studies, a mechanism for glyceraldehyde 3-phosphate dehydrogenase is proposed which appears to be applicable to the enzyme from all sources. This mechanism provides an explanation for many observations in the literature, particularly those relating to the difference in the catalytic properties of the free enzyme and its NAD$^+$ complex. The mechanism is consistent with most of the reported partial reactions catalyzed by the enzyme. A preliminary report of this work has previously appeared (12).

MATERIALS AND METHODS

Materials—Pea seeds (*Pisum sativum* L. var. Ida Blue and Ida Bell) were obtained from Dominion Food and Milling Ltd. Biochemicals, enzymes, and buffers were obtained from Sigma Chemical Co.; other chemicals were of the highest grade commercially available, except for ammonium sulfate, which met ACS specifications.

Assay of Glyceraldehyde 3-Phosphate Dehydrogenase—The assay used during purification contained, in a final volume of 3 ml, 1 mM NAD$^+$, 1 mM glyceraldehyde 3-phosphate, 20 mM potassium phosphate, 0.2 mM EDTA, 20 mM sodium fluoride, 3.3 mM cysteine, and up to 0.2 units of glyceraldehyde 3-phosphate dehydrogenase, in 133 mM Tris-chloride buffer, pH 8.75. The reaction was started by the addition of glyceraldehyde 3-phosphate, and NADH production at 25° was followed at 340 nm. Rates are expressed as the change in absorbance per min. There is an approximately linear relationship between the rate of reaction and enzyme concentration under these conditions. Modifications of this assay method are noted at the appropriate place in the text.

Analytical Methods—Protein was routinely estimated by the method of Lowry *et al.* (13) after precipitation of the protein with 5% trichloroacetic acid. The NAD$^+$ content of enzyme preparations was estimated by a modification of the method of Ferdinand (14). To 0.5-ml samples of enzyme was added an

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[1] The unit of enzyme activity is defined as the amount which will catalyze the formation of 1 μmole of NADH per min, under the conditions specified.

equal volume of 19 mM hydrochloric acid containing 1 mg per ml of pepsin (Sigma type "1:10,000 powder"). After 10 min, the solution was neutralized by the addition of 0.5 ml of 31 mM potassium carbonate and clarified by centrifugation. The NAD^+ concentration of the supernatant was estimated using ethanol and alcohol dehydrogenase (15).

SDS[2] gel electrophoresis was performed by the method of Shapiro *et al.* (16), except that 7% gels were used, all buffers were at pH 7.0, and gels and running buffers contained 0.1% 2-mercaptoethanol (v/v). Gels were stained and destained as described by Weber and Osborn (17). A standard curve was prepared using crystalline samples of catalase, fumarase, aldolase, and carbonic anhydrase. The mobility of each standard and of glyceraldehyde 3-phosphate dehydrogenase was determined, relative to a myoglobin internal marker.

Ultracentrifugation was performed on a Beckman model E analytical ultracentrifuge at 20° in unbuffered 0.2 M sodium chloride, pH 6.8. Sedimentation coefficients were converted to standard conditions (20°, water) as described by Schachman (18).

Amino acid analyses were performed on a Beckman 120C amino acid analyzer. Samples of NAD^+-free glyceraldehyde 3-phosphate dehydrogenase (19) were hydrolyzed for 24 hours using the procedure of Moore and Stein (20). Performate oxidation was performed by the method of Moore (21). For all amino acids except tyrosine, histidine, cysteine, and methionine, the composition was calculated from the mean of samples with and without performate oxidation. Tyrosine and histidine were determined only on samples that had not undergone performate oxidation, and cysteine was determined as cysteic acid after performate oxidation. Methionine was calculated from the mean of methionine sulfone and methionine, on samples with and without performate oxidation, respectively. No corrections were applied for incomplete hydrolysis or partial degradation of other amino acids.

RESULTS

Purification of Glyceraldehyde 3-Phosphate Dehydrogenase

Crude Extract—An acetone powder of pea seeds was prepared by the method of Hageman and Arnon (10) and extracted with 1.5 mM EDTA in 10 mM potassium phosphate buffer, pH 7.2, as described by these workers. The extract was clarified by centrifugation for 15 min at 10,000 × *g* and by filtration through glass wool. Unless otherwise stated, this EDTA-phosphate buffer was always used as a solvent for glyceraldehyde 3-phosphate dehydrogenase.

Heat Step—The heat step of Hageman and Arnon was used. Their description of this step is inadequate since the success of the method depends on the volume of enzyme extract, vessel geometry, stirring rate, etc. In the procedure we employed, the temperature of the crude extract was increased from 2° in a manner that approached 60° exponentially and took about 18 min to reach 55°, at which time the extract was rapidly cooled to 0°. This programmed temperature increase was achieved by immersing a beaker containing the crude extract in an electrically heated water bath. The temperature of the water bath was increased using a variable transformer, constantly monitoring the temperature of the enzyme solution.

This step only gives slight purification, but the heat-treated enzyme is more stable than the crude extract, possibly due to the inactivation of proteases in the extract. All subsequent steps were performed at 0–4°.

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

First Ammonium Sulfate Fractionation—A modification of the method of Hageman and Arnon was used. Solid ammonium sulfate (390 g per liter of heat-treated enzyme) was slowly added, the pH being maintained at 7.2 by the addition of 3.5 N ammonium hydroxide. After stirring for 30 min, the precipitate was removed by centrifugation at 10,000 × *g* for 15 min, and the enzyme was precipitated by the addition of 240 g of ammonium sulfate per liter of supernatant, again maintaining the pH at 7.2. The suspension was allowed to stand for 4 to 6 hours, and the enzyme was collected by centrifugation at 10,000 × *g* for 15 min. The pellet was dissolved in EDTA-phosphate buffer, pH 7.2, to give a protein concentration of about 30 mg per ml. The preparation was dialyzed for 90 min against 10 volumes of buffer, and again overnight against 10 volumes of fresh buffer. Without this dialysis, the second ammonium sulfate fractionation is unreliable.

Second Ammonium Sulfate Fractionation—The pH was adjusted to 7.8 with 3.5 N ammonium hydroxide and maintained at this value throughout this step. Solid ammonium sulfate (420 g per liter) was added and, after stirring for 30 min, the inactive protein was removed by centrifugation at 34,000 × *g* for 10 min. Solid ammonium sulfate (87 g per liter of supernatant) was added and, after stirring for 30 min, the enzyme was collected by centrifugation at 34,000 × *g* for 10 min. The enzyme was dissolved in buffer to give a protein concentration of about 20 mg per ml.

Ethanol Fractionation—Inactive protein was precipitated by slowly adding 700 ml of 95% ethanol at −20° per liter of enzyme solution, and the precipitate was removed by centrifugation at 34,000 × *g* for 5 min. A further 600 ml of ethanol per liter of supernatant was added, and the enzyme was collected by centrifugation at 34,000 × *g* for 10 min. The pellet was dissolved in enough buffer to give a protein concentration of about 4 mg per ml.

Acid Precipitation—The pH of the enzyme solution was adjusted to pH 5.1 with 1 N hydrochloric acid, the precipitate was removed immediately by centrifugation at 34,000 × *g* for 5 min and the supernatant was adjusted to pH 7.0 with 3.5 N ammonium hydroxide. If unusually good purification was achieved in the ethanol step, this acid step was less effective.

The purification is summarized in Table I, in which the data are an average of 16 purifications. The specific activity of the purified enzyme usually ranged from 200 to 230 units per mg of protein and never exceeded 240 units per mg. The enzyme

TABLE I

Purification of pea seed glyceraldehyde 3-phosphate dehydrogenase

The enzyme was purified as described in the text, taking samples at each stage in the procedure. These were assayed for enzyme activity and protein as described under "Materials and Methods." The data are an average of 16 purifications.

Fraction	Volume	Activity	Protein	Recovery	Specific activity	Purification
	ml	*units*	*mg*	%	*units/mg*	
Crude	690	10070	11730	100.0	0.858	1.00
Heated[a]	666	9320	7460	92.6	1.25	1.46
First $(NH_4)_2SO_4$	99	7480	2520	74.3	2.97	3.46
Second $(NH_4)_2SO_4$	50	6350	935	63.1	6.79	7.91
Ethanol	10.2	5220	39.1	51.8	134	156
Acid	10.1	3640	16.8	36.1	217	253

[a] Based on a sample which was removed and centrifuged at 34,000 × *g* for 15 min before assaying for protein and enzyme activity.

preparation could be stored for several weeks at 4° with only slight loss of activity.

NAD⁺ Content—Glyceraldehyde 3-phosphate dehydrogenase from many sources is isolated containing tightly bound NAD^+ (6). The NAD^+ content of several preparations of pea seed glyceraldehyde 3-phosphate dehydrogenase is shown in Table II. When the enzyme was precipitated with solid ammonium sulfate from a solution of 1 mM NAD^+, then washed twice with saturated ammonium sulfate, the precipitate contained 1 mole of NAD^+ per 35,700 g of protein.

The accuracy of these estimates of the NAD^+ content of the enzyme is dependent on the accuracy of the method of protein estimation. However, protein estimations on the purified enzyme were found to give similar values when the Lowry method (13), the biuret method (22), or the method of Murphy and Kies (23) were compared.

Structural Properties

After polyacrylamide gel electrophoresis at pH 8.9, using the method of Clarke (24), we were unable to demonstrate enzyme activity on the gels, although several staining procedures were tried. Subsequently, it was found that the enzyme is very unstable at alkaline pH values in the absence of substrates, which may account for this failure. Gel systems in the range of pH 6 to 7 were found to give unsatisfactory resolution, owing to the low mobility of the protein under these conditions. In SDS gels, only one protein band is seen (Fig. 1*A*), with a mobility of 0.618 to 0.621, relative to an internal myoglobin marker (Fig. 1*B*). This corresponds to a subunit molecular weight of 36,640 (Fig. 2).

In the analytical ultracentrifuge, the bulk of the material sedimented as a single symmetrical boundary, with an $s_{20,w}$ of 7.29×10^{-13} s at a protein concentration of 5.95 mg per ml and 7.56×10^{-13} s at 2.62 mg per ml. Under the same solvent conditions, the commercial rabbit muscle enzyme had an $s_{20,w}$ of 7.56×10^{-13} s at a protein concentration of 7.05 mg per ml. In the pea seed preparations, a small amount of lighter material was present, which may represent dimers or monomers. These experiments were performed in 0.2 M sodium chloride. Con-

TABLE II

NAD⁺ content of pea seed glyceraldehyde 3-phosphate dehydrogenase

Pea seed glyceraldehyde 3-phosphate dehydrogenase was purified as described in the text, and the NAD^+ content was determined as described under "Materials and Methods." The rabbit muscle enzyme was obtained from Sigma Chemical Co. and was treated in the same way as the purified pea seed enzyme.

Preparation	NAD⁺ content		
	Micromoles/g protein	Moles/mole enzyme[a]	
		Replicates	Mean
V and VI (pooled)	17.9	2.58	
	17.1	2.46	2.68
	20.8	3.00	
VII and VIII (pooled)	21.5	3.10	
	17.7	2.55	2.78
	18.8	2.71	
IX, X, XI, and XII (pooled)	14.6	2.10	
	15.6	2.25	2.13
	14.1	2.03	
XIII, XIV, XV, and XVI	19.7	2.84	2.79
(pooled)	19.0	2.74	
Rabbit muscle (commercial)	14.1	2.03	
	13.1	1.89	1.95
	13.4	1.93	

[a] A molecular weight of 144,000 was assumed.

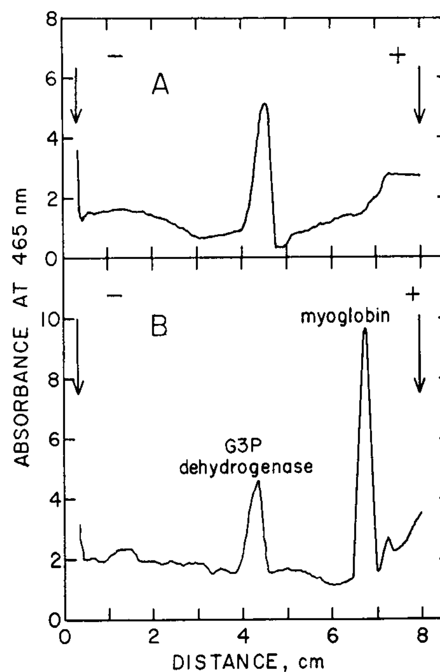

FIG. 1. SDS gels of pea seed glyceraldehyde 3-phosphate (*G3P*) dehydrogenase. Gels were run and stained as described under "Materials and Methods" and scanned using a Joyce-Loebl gel scanner. The curves shown are redrawn from these scans. In *A*, the sample contained 9 μg of pea seed glyceraldehyde 3-phosphate dehydrogenase, and in *B*, the sample contained 12 μg of myoglobin and 6 μg of pea seed glyceraldehyde 3-phosphate dehydrogenase. The *arrows* indicate the ends of the gels. The rather erratic base-line is also seen when gels are run in the absence of added protein and appears to be an artifact of the gel system.

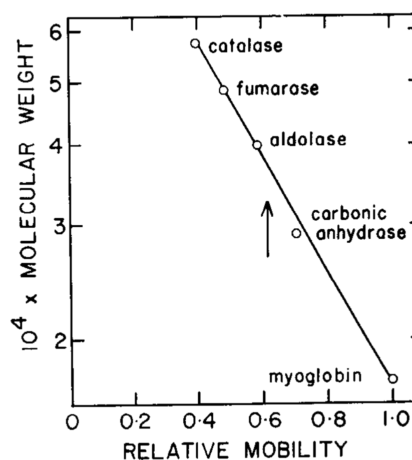

FIG. 2. Calibration curve for molecular weight determination using SDS-containing polyacrylamide gels. Gels were run and stained as described under "Materials and Methods," and the mobility of each protein subunit was measured, relative to myoglobin internal standards. The relative mobility of each protein is plotted against the log of its molecular weight. The variation between duplicate samples is contained within the size of the points. The relative mobility of pea seed glyceraldehyde 3-phosphate dehydrogenase is indicated by the *arrow* and corresponds to a subunit molecular weight of 36,640.

stantinides and Deal (25) have shown that similar concentrations of potassium chloride or ammonium sulfate may cause dissociation of the rabbit muscle enzyme into subunits and that the equilibrium is sufficiently slow for these components to be resolved in high speed ultracentrifugation experiments. A greater proportion of this lighter material was present in the more dilute pea seed preparation, which is consistent with this hypothesis. As only one protein band is seen in SDS gels, this interpretation of the minor component would appear to be correct.

The amino acid composition of pea seed glyceraldehyde 3-phosphate dehydrogenase is shown in Table III. For comparison, the composition of the enzyme from a number of other sources is also shown.

Kinetic Properties

The pH optimum for enzymatic activity was found to be in the range 8.5 to 9.0 (Fig. 3), and a pH of 8.75 was used for the assay. The enzyme from other sources has occasionally been reported to show kinetic anomalies such as secondary activation and cooperative interactions (26–28). The pea seed enzyme does not show such anomalies (29). However, each substrate, but especially glyceraldehyde 3-phosphate, was found to be inhibitory at high concentrations when the other substrates were present at less than saturating concentrations (Fig. 4). The purified preparation shows no activity when NAD^+ is replaced with $NADP^+$.

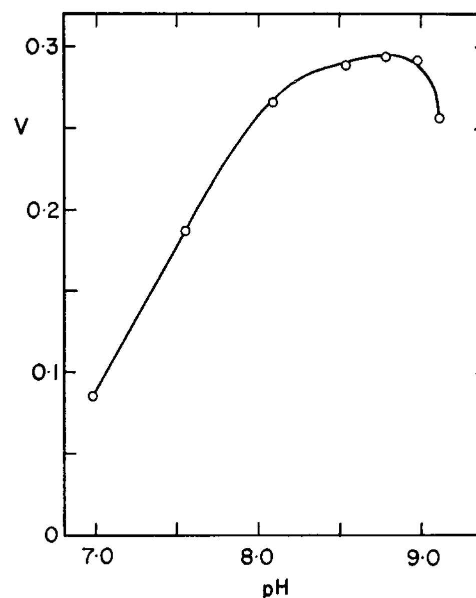

FIG. 3. Effect of pH on the activity of pea seed glyceraldehyde 3-phosphate dehydrogenase. Assays were performed as described under "Materials and Methods," except that the pH was varied as indicated. Each assay contained 1.4 μg of pea seed glyceraldehyde 3-phosphate dehydrogenase, purified to the ethanol fractionation stage.

TABLE III

Amino acid composition of glyceraldehyde 3-phosphate dehydrogenases

Pea seed glyceraldehyde 3-phosphate dehydrogenase was purified as described in the text, and the amino acid composition was determined as described under "Materials and Methods." The data for the enzyme from other organisms are taken from the literature.

Amino acid	Residues/1000[a]	
	Pea seed	Range of 20 species[b]
Lysine	97	76–91
Histidine	16	13–39
Arginine	39	25–34
Cysteine	7	6[c]–15
Aspartic acid	120	94–134
Threonine	70	49–84
Serine	68	45–80
Glutamic acid	69	49–76
Proline	35	28–46
Glycine	85	81–106[d]
Alanine	93	94–116
Valine	95	86–112
Methionine	14	12–31
Isoleucine	59	51–82
Leucine	67	55–66
Tyrosine	24	24–36
Phenylalanine	42	30–48

[a] Values for tryptophan have been omitted.

[b] The data are taken from Harris (2), Allison and Kaplan (6), and from Carlson and Brosemer (9).

[c] Allison and Kaplan (6) report 2 cysteines per 1000 residues for the sturgeon enzyme, but this is probably erroneously low.

[d] Allison and Kaplan (6) report 179 glycines per 1000 residues for the sturgeon enzyme, but this is probably a misprint.

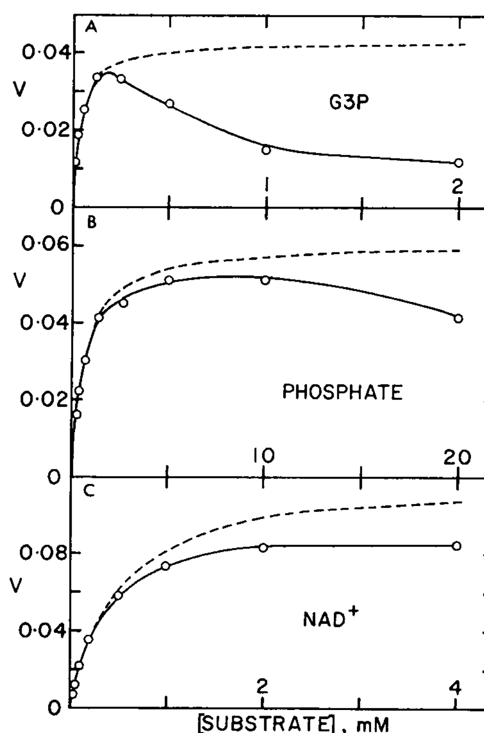

FIG. 4. Substrate inhibition of pea seed glyceraldehyde 3-phosphate (*G3P*) dehydrogenase. The enzyme was assayed as described by Duggleby and Dennis (29). In *A*, each assay contained 0.2 mM NAD^+, 0.72 mM phosphate, glyceraldehyde 3-phosphate as indicated, and 0.30 μg of purified enzyme. In *B*, each assay contained 0.2 mM NAD^+, 0.3 mM glyceraldehyde 3-phosphate, phosphate as indicated, and 0.35 μg of purified enzyme. In *C*, each assay contained 0.3 mM glyceraldehyde 3-phosphate, 0.72 mM phosphate, NAD^+ as indicated, and 0.65 μg of purified enzyme. The *solid lines* are fitted to the data by eye, and the *broken lines* represent an hyperbolic extrapolation of the data at low substrate concentrations (*i.e.* they are the lines which would be obtained if there were no substrate inhibition).

DISCUSSION

Previous purifications of glyceraldehyde 3-phosphate dehydrogenase from higher plants have given preparations with specific activities of the order of 10 units per mg of protein in yields of less than 20% (10, 11). In the present study, preparations were obtained with specific activities consistently in excess of 200 units per mg of protein, with a recovery of one-third of the initial activity. Allison and Kaplan (6) found the enzyme from the skeletal muscle of a variety of animals to have a specific activity of 20 to 25 units per mg, but the assay conditions they employed are probably suboptimal. Bloch *et al.* (30), using the refined purification techniques and improved assay of Ferdinand (14), obtained the enzyme from rabbit muscle with a specific activity of up to 194 units per mg. The pig muscle enzyme has been reported with a specific activity of 200 to 230 (31), that from lobster tail has a specific activity of 190 to 230 (32), and that of sturgeon has a specific activity of 180 to 220 units per mg of protein (32). The yeast enzyme is generally reported to have a specific activity of 150 to 160 (33–35), although Deal (36) gives figures from 130 to 390 units per mg of protein. Clearly, the specific activity of the pea seed enzyme is very similar to that from other sources, and this suggests that the preparation may be close to purity.

SDS gel electrophoresis and sedimentation velocity studies confirmed the purity of the preparation and suggest that the pea seed enzyme, like that from other sources, is a tetramer with a subunit molecular weight of 36,000 to 37,000. The finding that the enzyme is capable of binding 1 mole of NAD^+ per 35,700 g of protein is consistent with this and provides further evidence for the purity of the preparation. These data, together with the amino acid analysis, provide good evidence that the pea seed enzyme is similar to the enzyme from other sources in major structural respects.

Preliminary kinetic studies indicated an alkaline pH optimum and normal kinetics towards all three substrates. In these respects, the enzyme resembles that from other sources. Each of the substrates, particularly glyceraldehyde 3-phosphate and to a lesser extent phosphate, was found to be inhibitory at high concentrations when the concentrations of the other substrates are low. Glyceraldehyde 3-phosphate (37, 38) and phosphate (14) inhibition of the rabbit muscle enzyme have previously been reported.

Glyceraldehyde 3-phosphate dehydrogenase from pea seeds does not differ in any major respect from the enzyme from other sources. This has to be established since, in the following two papers, studies on the mechanism of the enzyme will be described. The mechanism which is postulated appears to be applicable to the enzyme from other sources and is consistent with much of the published data on glyceraldehyde 3-phosphate dehydrogenase.

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