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On the Existence of a Complex of Glycolytic Enzymes

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Abstract

It has been suggested in the literature that the glycolytic enzymes are organized into a multi-enzymic complex. We have evaluated this hypothesis for the phosphotriose-glycerate phosphate group of glycolytic enzymes of muscle using sucrose density gradient centrifugation, gel filtration, and ultrafiltration. Attempts were made to avoid dilution and changes in pH. The ratio of activities of the phosphotriose-glycerate phosphate group of enzymes was similar to that found in several other tissues that has led to their designation as a constant proportion group of enzymes. However, no evidence was obtained that they exist as a multi-enzymic complex in chicken breast muscle. As the pH of the press juice is raised to 7.0 and the temperature to 25°C, association occurs between some components in the muscle press juice as evidenced by a blocking of the pores of an ultrafiltration membrane. This association, however, does not involve the enzymes of the phosphotriose-glycerate phosphate group.

Introduction

Henning [1] defined a multi-enzyme complex as an "ordered association (not involving peptide linkages) of various enzymes which catalyze successive steps in a reaction sequence". The existence of a multi-enzyme complex in an intermediary metabolic sequence would add a high degree of order to the cell. It has been suggested that the enzymes of the phosphotriose-glycerate phosphate group (PTG) are organized into a multi-enzyme complex [2-4]. This sequential group of enzymes includes triose phosphate isomerase (TMI), glyceraldehyde phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), and enolase (EN). Pette *et al.* [5] were the first workers

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to show that the activities of the enzymes of the PTG group had a constant ratio from different muscle sources. Mier and Cotton [2] found that the activity pattern measured by Pette *et al.* would indicate that the enzymes are present in equimolar amounts. These latter workers hypothesized that the synthesis of this group of enzymes was controlled by a single operator gene, and that during or upon completion of their synthesis, the five enzymes were assembled into a multi-enzyme complex. Kraus *et al.* [4] also presented evidence for the possible coordination of synthesis of the PTG group of enzymes at the genetic level from their observations after treatment of hypothyroid rats with I^{131} and triiodothyronine. The five enzymes were synthesized in the proportion needed for formation of the complex.

Ginsburg and Stadtman [6] have recently reviewed the physical and biochemical properties and the procedures for the detection and identification of multi-enzyme complexes formed from the "soluble" enzymes of the cell. Multi-enzyme complexes have generally been detected by standard protein separation techniques such as filtration on a Sephadex column [7], chromatography on DEAE cellulose [8] or centrifugation through a sucrose density gradient [9]. Changes in enzymic activity in the presence or absence of another enzyme [10, 11] or changes in the catalytic efficiency of a sequential process [12] have also provided indications for the existence of multi-enzyme complexes.

The finding of glycolytic enzymes in association with particulate fractions of the cell [3,13] or highly localized concentrations of the glycolytic enzymes in the cell [14, 15] have also led to speculations concerning the assemblage of these enzymes into complexes. In this paper, we report experiments which were carried out to determine whether the enzymes of the PTG group exist as a multi-enzyme complex.

Materials and Methods

Materials

Reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD^+), adenosine triphosphate (ATP), and adenosine diphosphate (ADP) were purchased from P-L Biochemicals Inc. Reduced glutathione, phosphoglyceric acid, sodium pyruvate, pyruvate kinase, phosphoglycerate kinase, and α-glycerophosphate dehydrogenase were products of Calbiochem. Glyceraldehyde-3-phosphate, 2-phosphoglycerate, glyceraldehyde phosphate dehydrogenase, enolase, and lactate dehydrogenase were obtained from Sigma Chemical Co. All other chemicals were the purest available commercially.

Biological Source of Glycolytic Enzymes

Chickens (female) were supplied from the flock maintained by the Department of Veterinary and Animal Sciences at the University of

Massachusetts. The chickens were from mixed breeds and of various ages. The birds were provided *ad libitum* with a commercial feed. Unless otherwise stated, the chickens were sacrificed by injection of air into the heart. Following sacrifice, the breast muscle (*Pectoralis major*) was immediately excised and placed in ice-cold, de-ionized distilled water. Unless otherwise stated, all subsequent operations were carried out at 0-4° C. Connective tissue and excessive deposits of fat were removed from the muscle.

Preparation of Homogenates and Press Juice

A whole homogenate was prepared by blending the excised chicken breast muscle in distilled de-ionized water for 1 min in a Waring Blender at full speed. The whole homogenate was then centrifuged in the No. 40 rotor of a Spinco model L-2 Ultracentrifuge at 40,000 rpm for 30 min. The sedimented fraction was re-homogenized in 15 mM histidine buffer, pH 7.0 in a volume equal to the decanted supernatant fraction. The percentage of enzyme retained on the particulate fraction was determined by assaying for enzymatic activity in the supernatant and sedimented fractions. Recoveries were always determined to insure that the results were not due to inactivation of enzymes.

For preparation of the press juice, chicken breast muscle was diced into 1 cm cubes. These pieces were packed into cellulose nitrate centrifuge tubes and centrifuged in the No. 40 rotor at 36,000 rpm in a method similar to that of Amberson [16].

Sucrose Density Gradient Centrifugation

Sucrose density gradients were prepared by a method similar to that of Martin and Ames [17]. A linear gradient of 5 to 22% sucrose was formed. The sucrose gradients were allowed to stand for at least 2 h at 4° prior to use. All of the gradients were buffered to pH 7.0 with 15 mM histidine buffer unless otherwise stated. 0.1 ml of enzyme solution was layered on top of the gradients. The sucrose density gradients were then centrifuged in the SW-39 swinging bucket rotor at 39,000 rpm for 20 h after which 0.4 ml fractions were collected and assayed.

Sephadex Filtration

Type G-100 Sephadex (Pharmacia Fine Chemicals Inc.) was chosen for gel filtration studies since its fractionation range for globular proteins is between molecular weights of 4,000 and 150,000. The opened pores were thus similar in size to GAPDH, which is the largest of the PTG group of enzymes. Blue dextran was used to follow the course of elution.

Ultrafiltration

Separation by ultrafiltration was achieved with Diaflo ultrafiltration membranes (XM-100 type from Amicon Corp.) using a Model No. 52 cell chamber. The chamber was subjected to pressure (10 psi) from a source of compressed N_2 gas. This membrane will retain 20% of aldolase (molecular weight = 142,000) and 90% of gamma globulin (molecular weight = 160,000).

Enzymic Assays

The activities of all enzymes were determined spectrophotometrically by measuring the oxidation of NADH to NAD^+ at 340 nm. Readings were taken every 15 sec for 2 min at 28° C and pH 7.6. Reactions were initiated with the enzyme. The assay conditions were slight modifications of those described by Rassner [18] and Shonk and Boxer [19]. The assay systems contained as follows:

Triose phosphate isomerase: 50 mM triethanolamine, 5 mM EDTA, 0.15 mM NADH, 2 mM glyceraldehyde-3-phosphate, and one unit of α-glycerophosphate dehydrogenase.

Glyceraldehyde phosphate dehydrogenase: 50 mM triethanolamine, 5 mM $MgSO_4$, 0.15 mM NADH, 2.5 mM reduced glutathione, 2.5 mM ATP, 8 mM 3-phosphoglyceric acid, and two units of phosphoglycerate kinase.

Phosphoglycerate kinase: 50 mM triethanolamine, 5 mM EDTA, 10 mM $MgCl_2$, 0.15 mM NADH, 2.5 mM reduced glutathione, 2.5 mM ATP, 8 mM 3-phosphoglyceric acid, and two units of glyceraldehyde phosphate dehydrogenase.

Phosphoglycerate mutase: 50 mM triethanolamine, 5 mM EDTA, 10 mM $MgCl_2$, 0.15 mM NADH, 2.5 mM ADP, 8 mM 3-phosphoglyceric acid, two units of enolase, two units of pyruvate kinase, and two units of lactate dehydrogenase.

Enolase: 50 mM triethanolamine, 5 mM EDTA, 10 mM $MgCl_2$, 0.15 mM NADH, 2.5 mM ADP, 4 mM 2-phosphoglyceric acid, two units of pyruvate kinase, and two units of lactate dehydrogenase.

Protein Determination

Protein concentrations were determined by the biuret method, as described by Gornall *et al.* [20].

Results

Quantitative Assessment of the PTG Group of Enzymes in Chicken Skeletal Muscle

In the first line of Table I, the activities of the five enzymes relative to GAPDH in a 10% whole homogenate are given. The numbers in

TABLE I. Distribution of the PTG group of enzymes between supernatant and sedimented fractions

	GAPDH (100)	PGK (82)	PGM (58)	EN (30)	TIM (1900)
10% whole homogenate (relative to GAPDH)	100	55	60	39	910
% in sediment	40	7	3	8	4
Press juice preparation					
Press juice (relative to GAPDH)	100	60	57	42	1200
Pressed muscle (relative to GAPDH)	100	39	51	38	780

parentheses represent the activity pattern for equimolar amounts as calculated by Mier and Cotton [2]. Our results agreed fairly closely with these authors though a difference does exist for TIM. However, the activity of TIM is within the activity pattern range found by Pette *et al.* [5]. The PTG group of enzymes appears to be in or near equimolar amounts in chicken breast muscle.

The second line in Table I gives the activity profile of the five enzymes of the PTG group after centrifugation of the 10% whole homogenate. The amount of the different enzymes associated with the sedimented fraction vary markedly with the greatest amount being that of GAPDH.

The last two rows of data in Table I represent the activities of the five enzymes relative to GAPDH in the press juice and pressed muscle fractions obtained after centrifugation of the diced muscle. Such a method does not involve dilution of the muscle tissue during extraction of the enzyme. These indicate that by this procedure the PTG group of enzymes in the extracted press juice were in nearly equimolar amounts.

This press juice was investigated for the multi-enzyme complex of the PTG group since the mole ratios were similar to those of the whole muscle.

Fractionation of the Press Juice

Density gradient centrifugation gave the activity pattern shown in Fig. 1 for three of the enzymes of the PTG group, namely, GAPDH, PGK, and enolase. A clear-cut separation pattern was observed with the sedimentation rate related to molecular weight as expected if the enzymes were not associated.

This technique was used to determine if a series of purified enzymes could associate into a complex as ionic strength was varied from 0 to 0.15 and pH was varied from 6.5 to 8. The cofactors ADP, ATP, NAD^+, NADH, and magnesium chloride were added at different concentrations and in different combinations to the enzyme solutions and the gradients.

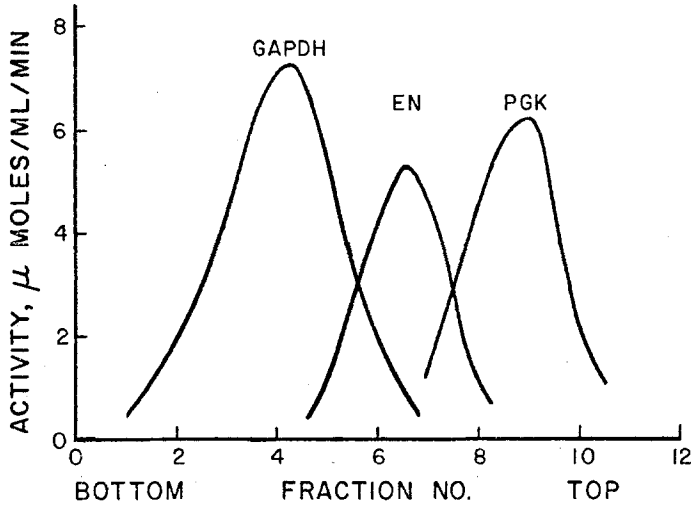

Figure 1. Activity pattern of glyceraldehyde phosphate dehydrogenase (GAPDH), enolase (EN), and phosphoglycerate kinase (PGK) after density gradient centrifugation of press juice prepared from chicken breast muscle on a linear gradient of 5 to 22% sucrose. Details of the procedure are given in the text.

Phospholipids were also added. The centrifugation patterns were similar in all cases to that seen in Fig. 1.

Assays of the activity of the effluent following blue dextran from a Sephadex gel filtration column are shown in the first row in Table II. The activities are expressed relative to GAPDH. The enzymes were not present in equimolar amounts in this fraction. GAPDH was the enzyme in highest concentration which is consistent with the proposition that the enzymes move through the column as separate entities rather than a complex.

For the ultrafiltration studies, the membrane XM-100 was chosen, since the individual enzymes of the PTG group should pass through this unhindered. If the enzymes exist in a complex, however, they should be retained in the press juice. We assayed the activities of the enzymes in the first 0.5 ml of filtrate (a small quantity was chosen to prevent the build-up of protein on the membrane and the stoppage of the filtration rate of the proteins). If the enzyme passed freely through the filter, the activity in the filtrate should be equal to that in the press juice and the ratio should equal 1. The results are shown in the second row of Table 2 where it is seen that all of the enzymes of the PTG group have a ratio close to 1 which indicates that they do not exist as a complex.

In other experiments, the press juice was centrifuged for five hours at 48,000 rpm in a No. 50 rotor in the Spinco model L-2 ultracentrifuge.

TABLE II. Fractionation of the press juice

	GAPDH	PGK	PGM	EN	TIM
(a) Sephadex filtration (relative to GAPDH)	100	8	16	6	58
(b) Ultrafiltration (press juice/ultrafiltrate)	1.09	0.90	0.99	1.06	1.04
(c) Ultracentrifugation					
(a) % sediment	16	5.0	5.0	3.0	8.0
(b) % sediment (plus washed particles)	10	4.5	4.0	5.0	5.2

The purpose was to use a greater g force than that involved in the preparation of the press juice and to determine if this greater g force would sediment a complex of PTG enzymes. Again, however, a greater sedimentation was noticed for the enzyme of highest molecular weight, GAPDH. Addition of washed chicken breast muscle particles (prepared from a tissue homogenate) to the press juice prior to centrifugation had no effect on the sedimentation of the five enzymes. The washed muscle particles were added in the event that a structural system of the muscle was necessary to maintain the integrity of the protein complex.

Prevention of pH Drop after Sacrifice

The pH of the chicken breast muscle drops quickly after death and the pH of the press juice preparation was usually about 5.9. We felt that perhaps the low pH might be responsible for the dissociation of the complex of the PTG enzymes; therefore, procedures were instituted to control the drop in pH. These included intravenous injection with iodoacetate or epinephrine after fasting for 30 hours as described by de Fremery and Pool [21]. Another technique used was to sacrifice the chickens with sodium pentobarbitol. The muscle was excised immediately, placed in liquid nitrogen, and then freeze-dried. The dried muscle pieces were placed in solutions of either 0.1 M iodoacetate or in 0.2 M sodium fluoride with imidazole buffer at pH 7.0 or 7.2. By this technique there is no dilution of cytoplasmic proteins. The efficacy of these procedures in controlling the pH of the press juice is shown in Table III. Intravenous injection of iodoacetate or epinephrine had only a moderate effect on the pH of the press juice. The freezing imbition procedure, however, was quite successful. When these press juices were examined by the ultrafiltration technique for the ratio of enzyme passing through the filter to that in the original solution, the results were all very close to 1.0 indicating that the complex was not present.

pH Adjustment of Press Juice

The pH of the press juice was raised to 7.0 by the addition of 1 M imidazole. This required addition of a volume equal to about 10% of the

TABLE III. Treatments to prevent pH drop

Treatment	pH of press juice
(1) No treatment	5.9
(2) Intravenous injection of iodoacetic acid (0.1 gm/pound body weight)	6.2
(3) Starvation for 30 h, intravenous injection of epinephrine (0.5 gm/pound body weight)	6.3
(4) Sacrifice with Na pentobarbital (130 mg); freeze sliced muscle in liquid nitrogen; dry under vacuum (70 u, −20°C, 4 h); imbibe in 100 mM iodoacetic acid (pH 7.0)	6.9
(5) Sacrifice with Na pentobarbital (130 mg); freeze sliced muscle in liquid nitrogen; dry under vacuum (70 u, −20°C, 4 h); imbibe in 0.2 M NaF (pH 7.2)	7.1

press juice. The sample was then held at room temperature for 1.5 h after which it was subjected to ultrafiltration at room temperature. Results are presented in Table IV along with results at 4° C and pH 6 as well as the other combinations of these pH values and temperatures. It is obvious that raising the pH and the temperature (especially together) increases the ratio of enzyme activity in the press juice to the filtrate. This result could be due to the formation of a multi-enzyme complex or to the aggregation or formation of some other material which was plugging the pores in the filter.

To test this, press juice was centrifuged at 50,000 rpm in the No. 50 rotor for 5 h. Three fractions were obtained, a colorless upper portion comprising about 15% of the sample, a red middle section comprising about 80% of the press juice, and a red gummy translucent sedimented fraction. In Table V is shown the ultra-filtration data for a normal press juice at pH 6 and 4°, and the press juice in which the temperature and pH had been raised (Experiments 1 and 2, respectively). The increase in the ratio of activity in the press juice to the ultrafiltrate can again be observed. After centrifugation, the enzyme ratios of the middle clear layer of the sample in which the pH had been adjusted to 7 and

TABLE IV. Effect of temperature and pH changes on ultrafiltration of the press juice

	(Press juice/ultrafiltrate)			
	RT[a], pH 7	RT, pH 6	4°C, pH 7	4°C, pH 6
GAPDH	8.2	2.0	2.8	1.9
PGK	4.0	1.5	2.8	1.9
EN	6.4	1.7	2.6	2.0
Protein	2.4	1.5	1.6	1.5

[a]RT: room temperature

TABLE V. Ultrafiltration of the press juice after recentrifugation

Experiment	Condition	(Press juice/ultrafiltrate) GAPDH	PGK	EN
1	press juice; pH 6, 4°C	2.0	2.1	2.0
2	press juice; pH 7, RT	50.5	17.6	22.8
3	sample 2, centrifuged for 5 h at 50,000 rpm at 25°C, minus the sediment and upper clear layer	1.8	1.7	1.6
4	sample 3, upper clear layer added back	7.3	3.9	4.7
5	upper clear layer in water, then sample 3	48.6	25.2	16.2
6	upper clear layer in water, then sample 1	16.1	15.1	13.9
7	clear layer (prepared from sample 1) in water, then sample 1 minus its upper clear layer	2.3	2.5	2.4

incubated at room temperature were reduced to what they had been with the pH 6, 4° sample (Experiment 3, Table V). In the fourth experiment, the upper clear layer was added back to the clear red fraction, and the ratios once again increased. This was even more evident in an experiment in which the upper clear layer was mixed with water and filtered and then the sample from Experiment 3 was filtered (Experiment 5). The same thing is shown in Experiment 6 except that sample 1 was filtered after the clear upper layer in water had been first subjected to the filtering steps. It gave a high ratio.

The clear layer could also be prepared by centrifugation of the press juice at pH 6 and 4°. When this clear layer was placed in water and filtered through the membrane followed by sample 1 minus its upper clear layer, there was little or no effect (Experiment 7). Whatever was present in the upper clear layer to block the pores, required the higher pH and temperature to form. It is evident from this that the high ratios observed in the pH- and temperature-adjusted samples was not due to a multi-enzymic complex of the PTG enzymes but due to interference by other compounds which had formed due to the change in pH and temperature. Centrifuging the upper clear layer after mixing with Ringer's solution gave results comparable to those in which the upper clear layer was taken up in deionized distilled water.

Discussion

No evidence was obtained in the experiments reported in this paper for the existence of a multi-enzymic complex composed of the five enzymes of the PTG group. The roughly equimolar amounts of enzymes found in the press juice and the pressed muscle as shown in Table I could have been due to the fact that not all of the water is extracted from the cell

by this technique. The difference in the percentage of enzyme extracted and water extracted from the cell is not very large.

Attempts were made to keep the procedures used in handling the extracts as close to physiological as possible and to avoid all procedures which might tend to disrupt a labile enzymic complex. Gel filtration on Sephadex and sucrose density gradient centrifugation have the disadvantage that the enzymes become diluted during the operation. Ultracentrifugation of the press juice and the ultrafiltration technique, which would not lead to dilution, also gave no evidence for the existence of a multi-enzymic complex of this group of enzymes.

pH control of the press juice was achieved, but the use of this technique also did not give any evidence for the existence of a complex. Adjustment of the pH of the press juice to pH 7 and/or warming it to room temperature did not cause reaggregation of the PTG group of enzymes. The formation of some other material had occurred which was capable of blocking and clogging the pores of an ultra-filtration membrane after the pH and temperature adjustment.

Our data does not support the hypothesis of the existence of a multi-enzyme complex of the PTG group of enzymes. However, it is possible that even the treatments we used were not sufficiently mild to prevent disruption of such a complex. deDuve [22] also did not find any evidence for a multi-enzyme complex of the glycolytic enzymes in the cytosol of rat liver cells. Hübscher *et al.* [23] concluded that since there were only one to two molecules of phosphofructokinase for every 100 molecules of aldolase and TIM in the rat liver cell, it was unlikely that all the glycolytic enzymes existed in an "ideal" complex. They did not rule out the possibility that some of the glycolytic enzymes form stoichiometric complexes.

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