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SOLUBILIZATION AND PARTIAL PURIFICATION OF SARCOPLASMIC RETICULUM-BOUND HEXOKINASE OF FROG SKELETAL MUSCLE

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SUMMARY

The sarcoplasmic reticulum-bound hexokinase of frog skeletal muscle has been solubilized and purified (PS) 60-fold. The molecular weight as obtained by calibrated Sephadex G-200 gel column and sucrose gradient analysis was 112 875 ± 5866 and 95 212 ± 2740 respectively. The propensity for molecular aggregation was considerable.

The purified enzyme (PS) was rebound to enzyme-denuded sarcoplasmic reticulum. The kinetics of the original bound, purified unbound and purified rebound hexokinase was examined with respect to orthophosphate activation and inhibition with 1,5-anhydroglucitol-6-*P* (analogue of glucose-6-*P*).

At optimum orthophosphate concentration, activation was biphasic in the presence of varying MgATP for the original bound hexokinase. Thus orthophosphate activation at 0.5 and 10 mM MgATP was 90 and 100%, respectively. Solubilization and purification of the original bound hexokinase resulted in loss of the biphasic response with activation at high MgATP concentration only. Rebinding of the PS hexokinase to enzyme-denuded sarcoplasmic reticulum restored the biphasic activation in the presence of MgATP.

The original sarcoplasmic reticulum-bound hexokinase was considerably more resistant to 1,5-anhydroglucitol-6-*P* inhibition with respect to MgATP, compared to the PS hexokinase. Rebinding of PS hexokinase to enzyme-denuded sarcoplasmic reticulum restored the resistance to 1,5-anhydroglucitol-6-*P* inhibition. Thus the K_i for bound, unbound and rebound enzyme was 0.08, 0.026 and 0.11 mM respectively.

Binding of hexokinase to sarcoplasmic reticulum ($1/K_{\text{dissociation}}$) was optimal at pH 6.5 and 8.5. The dissociation constant, K_D for original bound hexokinase at pH 6.5, 8.5 and 7.5 was 0.48, 0.54 and 63.4 units/ml, respectively.

INTRODUCTION

The 105 000 × *g* soluble and sarcoplasmic reticulum-bound hexokinase(s) of frog skeletal muscle have been previously characterized and compared with respect to kinetic and other properties[1-3]. The particulate enzyme had a one-fifth lower K_m for MgATP, was activated 1.6-fold by orthophosphate, and was 3 times more resistant to the competitive inhibition of 1,5-anhydroglucitol-6-*P* (analogue of glucose-6-*P*) with respect to MgATP[2]. The original soluble enzyme (OS) was noted to undergo aggregation, particularly at low ionic strength[3]. Aggregation (or low ionic strength) appeared to confer new properties on the soluble enzyme, *i.e.* activation by orthophosphate and resistance to inhibition by 1,5-anhydroglucitol-6-*P*[3].

In the present report, the sarcoplasmic reticulum-bound hexokinase has been solubilized and purified 60-fold. The purified solubilized particulate enzyme (PS) has been characterized with respect to molecular weight, propensity for molecular aggregation and binding properties. The bound, unbound and rebound hexokinase has been analyzed with respect to K_m for MgATP, K_m for glucose, activation with orthophosphate and inhibition with 1,5-anhydroglucitol-6-*P*. Evidence is presented for the cellular organization and regulation of frog skeletal muscle hexokinase.

EXPERIMENTAL

Female *Rana pipiens* were obtained from Lake Champlain Frog Farms, Alburg, Vermont, and handled as described previously[2,3]. Hexokinase measurements were performed on preparations obtained from total hindleg skeletal muscle.

Preparation and purification of sarcoplasmic reticulum-bound hexokinase

Crude particulate hexokinase was prepared as described previously[2], in 50 mM Tris buffer, pH 7.5, 5 mM EDTA, 5 mM mercaptoethanol. This material was freeze dried and stored at −70°. The freeze dried powder was resuspended in one-half its original volume with 500 mM Tris buffer, pH 7.5, 5 mM EDTA, 5 mM mercaptoethanol, 100 mM glucose by the assistance of a Dounce homogenizer. This suspension was then centrifuged for 1 h at 105 000 × *g* in a Model L-2 Beckman Ultracentrifuge. All centrifugations were performed at 4°. The supernatant, S1 was decanted and saved. The resultant particulate fraction was resuspended a second time in one-half the same diluent as above and similarly recentrifuged. The supernatant S2 was decanted and combined with S1.

$(NH_4)_2SO_4$ fractionation of combined S1 and S2 was performed at 0° with finely ground $(NH_4)_2SO_4$ powder. This was added to the vortex of a continuously stirred enzyme solution (teflon-coated magnetic stirring bar). The first $(NH_4)_2SO_4$ fraction consisted of the addition of 12 g/100 ml of solution. The succeeding seven fractions consisted of the addition of 4 g/100 ml each. At each step in the fractionation, the precipitate was allowed to form for 5 min and then separated by centrifugation at 4000 × *g* for 5 min. The various precipitates were dissolved in the smallest possible volume of 50 mM Tris buffer, pH 7.5, 5 mM mercaptoethanol, 100 mM glucose. This resulted in a turbid solution which was clarified by centrifugation. The best fractions were combined (usually Fractions 3 through 8). The average of approximately 30 purifications is given in Table I. Glucose was omitted from the enzyme

TABLE I

PURIFICATION OF PARTICULATE HEXOKINASE

	Vol. (ml)	*Protein (mg)*	*Units**	*Specific activity (units/mg protein)*	*Purification*	*Recovery (%)*
4000 × *g* homogenate	288	2888	32 634	11.3	—	100
Particulate subfraction freeze dried S1 + S2	144	268	8 150	30.4	2.7	25
eluates $(NH_4)_2SO_4$ (20–40%, w/v)	144	117	12 075	103.2	9.1	37
	6	49	3 263	665.9	58.9	10

* nmoles/min per ml.

solution when glucose K_m's were to be obtained. The enzyme solution was also dialyzed for 24 h at 4° against the buffer solution (1 ml enzyme per 1 buffer). Glucose K_m's were determined as described previously[3].

Binding of purified hexokinase to sarcoplasmic reticulum

Stored sarcoplasmic reticulum (−70°) was denuded of hexokinase by repeated solubilization as above, *i.e.* S3, S4, *etc.* (similar to preparation of S2), until hexokinase activity was no longer detectable. The final pellet was resuspended in 50 mM Tris buffer, pH 7.5, 5 mM mercaptoethanol at a protein concentration of 6 mg/ml. Equal volumes of the purified enzyme and the denuded sarcoplasmic reticulum were combined, suspended with the aid of a Dounce homogenizer, and kept at 0 or 30° for 10 min. (Similar results were obtained at either temperature.) The suspension was then centrifuged at 105 000 × *g* for 1 h. The supernatant was discarded and the particulate fraction resuspended in its original volume of buffer. The hexokinase activity of the rebound sarcoplasmic reticulum averaged 40% of the original enzyme activity to which it was exposed.

Solubilization of original sarcoplasmic reticulum-bound hexokinase

S1 was obtained from reconstituted crude particulate hexokinase following dialysis overnight at 4° in 5 mM EDTA, 5 mM mercaptoethanol, 50 mM Tris buffer at pH 6.5, 7.5 or 8.5 (1 ml enzyme suspension per 1 buffer). The enzyme suspension was then sedimented at 105 000 × *g* for 1 h and the supernatant designated S1. The sediment was resuspended in its original volume and designated P1. This suspension was then resedimented as above and S2P2 isolated. S3P3 and S4P4 were similarly obtained. pH was tested prior to and following each isolation step. No significant alterations in pH were noted. A dissociation constant, K_D is defined as $(P \cdot E)/PE$ where PE is equal to the units of bound enzyme activity, E equals the units of solubilized activity and P equals the available unbound sites on the particle in terms of enzyme units of activity which are capable of being bound.

Gel filtration estimation of molecular weight was performed by the method of ANDREWS[4] with Sephadex G-200 as described previously[3].

Sucrose gradient estimation of molecular weight was performed by the method of MARTIN AND AMES[5] with a 5 to 20% continuous sucrose gradient as described previously[3].

Hexokinase assay

Hexokinase activity was measured at 29° by a coupled system which measured the formation of NADPH at 340 mμ. The assay cuvette contained 4 μg/ml glucose-6-*P* dehydrogenase, 0.46 mM $NADP^+$, 20 mM glucose, equimolar MgCl + ATP concentrations (10–20 mM) and 20–100 μl/ml enzyme containing 40–200 μg/ml protein in Tris buffer, pH 7.5, 5 mM mercaptoethanol. Enzyme rates were linear, directly proportional to concentration of enzyme and zero in the absence of glucose or ATP.

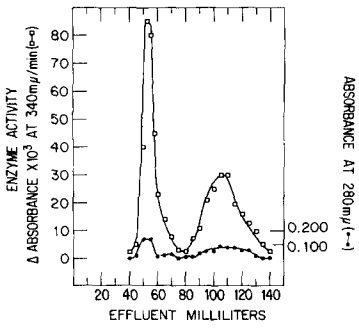
Protein was measured by the method of LOWRY *et al.*[6].

Materials

Distilled, deionized water was used at all times. Enzymes and reagents were purchased from vendors as described previously[2,3]. The cyclohexylamine salt of 1,5-anhydroglucitol-6-*P* was prepared by enzymatic phosphorylation of 1,5-anhydroglucitol[7] which was obtained through the generosity of Dr. R. K. Crane (Rutgers Medical School, New Brunswick, N.J.). Sucrose was obtained from Merck and Co. All other reagents were obtained from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim Corp., New York.

RESULTS

The molecular weight of the purified particulate hexokinase (PS) as estimated by calibrated Sephadex column was 112 875 ± 5866 (S.E., 9 experiments), and



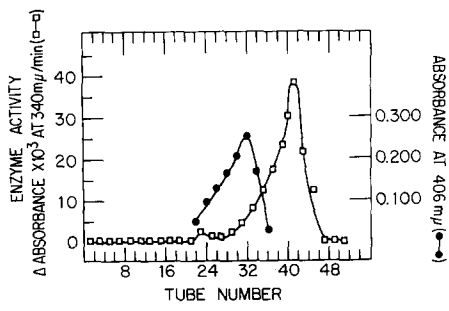


Fig. 1. Sephadex G-200 gel filtration of solubilized, purified sarcoplasmic reticulum-bound hexokinase. Enzyme preparation and column were equilibrated with 50 mM Tris buffer, pH 7.5, 5 mM EDTA, 5 mM mercaptoethanol, 100 mM glucose. The calibrated column revealed molecular weights of 115 000 and 550 000.

Fig. 2. Sucrose gradient analysis of solubilized, purified sarcoplasmic reticulum-bound hexokinase. Purified hexokinase, 0.5 mg, plus catalase 2.3 mg, ●—●, in a volume of 0.2 ml was layered onto a 5–20% continuous sucrose gradient in 50 mM Tris buffer, pH 7.5, 5 mM EDTA, 5 mM mercaptoethanol. The gradient was centrifuged at 37 000 rev./min in a Beckman L2 Ultracentrifuge for 8 h at 4°. See EXPERIMENTAL for further details. The molecular weight was estimated to be 191 750 from the reference position of catalase.

similar to the 105 000 reported previously for OS hexokinase[3]. Of interest was the marked propensity for molecular aggregation on Sephadex G-200 which varied from 50 to 88% as estimated from the integrated areas of the curves. This was considerably more than the 15% molecular aggregation noted for purified OS hexokinase[3]. A typical example is illustrated in Fig. 1 where the two hexokinase species had estimated molecular weights of 115 000 and 550 000.

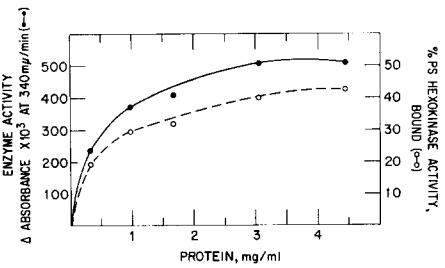


Fig. 3. Binding of solubilized purified particulate hexokinase to enzyme-denuded sarcoplasmic reticulum. Total hexokinase activity bound (●—●,) as well as percent of total enzyme activity bound (○—○) is plotted against sarcoplasmic reticulum protein concentration.

The molecular weight ($s_{20,w}$), as estimated by sucrose gradient analysis was 95 212 ± 2740 (2.7S) (S.E., 4 experiments) similar to the 99 700 reported for OS hexokinase[3]. Similar molecular aggregation as noted above, was also obtained with sucrose gradient analysis. Thus, in 4 out of 8 determinations the enzyme was present completely in the aggregated form, Fig. 2. In this figure the estimated molecular weight was 191 750 ($s_{20,w}$ = 7.5 S). Again this degree of aggregation for the PS hexokinase was considerably more extensive than that reported for the OS hexokinase[3].

Binding of the purified particulate hexokinase to sarcoplasmic reticulum

Binding experiments (see EXPERIMENTAL) revealed the feasability of studying rebound PS hexokinase. In Fig. 3, enzyme activity bound, as well as percent of total activity bound, is plotted against sarcoplasmic reticulum protein concentration. A concentration of 3 mg/ml protein was found to be optimal and was employed for all kinetic experiments.

Activation with orthophosphate

The activation kinetics of increasing orthophosphate concentration at constant glucose, but varying MgATP concentration is depicted in Fig. 4, for the original bound (Fig. 4A), unbound purified PS hexokinase (Fig. 4B) and rebound hexokinase (Fig. 4C). Similar results were obtained in several experiments. The particulate enzyme showed activation at all MgATP concentrations with a biphasic maximum effect at 0.5 and 10 mM MgATP of approximately 90 and 100% activation respectively (see insert Fig. 4A).

Solubilization and purification of the particulate hexokinase abolished activation with orthophosphate at low MgATP concentration, *i.e.* 0.5 and 1 mM MgATP. Activation was present at 2.5 and 7 mM MgATP and optimum at 10, 15 and 20 mM MgATP (approximately 50%, see insert Fig. 4B). It is to be recalled that the OS

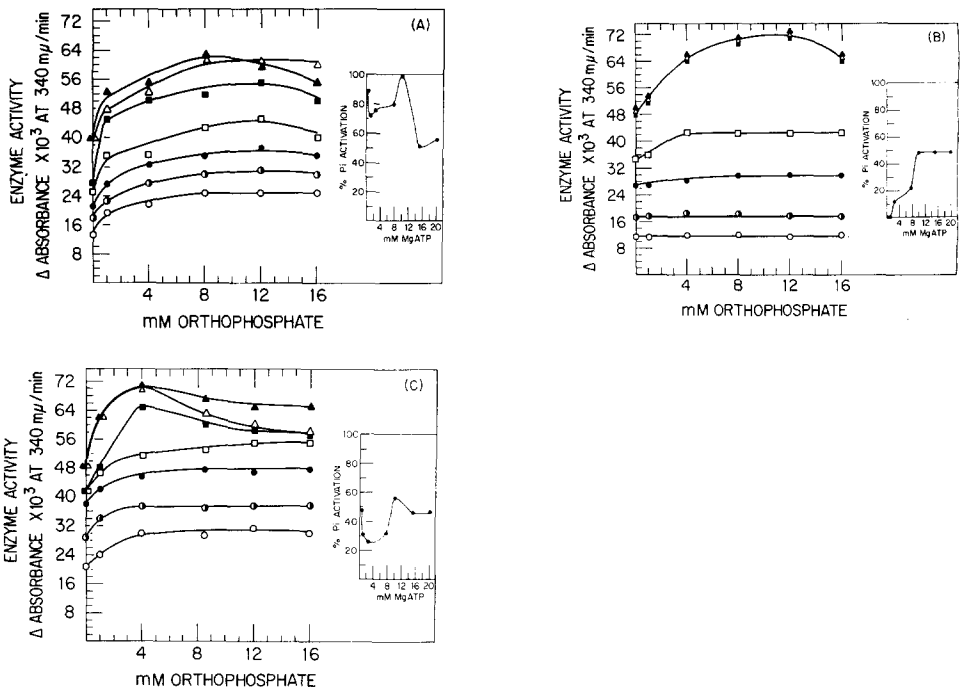


Fig. 4. A. Orthophosphate activation of original sarcoplasmic reticulum-bound hexokinase at varying levels of MgATP: ○, 0.5 mM; ◑, 1 mM; ●, 2.5 mM; □, 7.5 mM; ■, 10 mM; △, 15 mM; and ▲, 20 mM. Insert refers to optimum P_i activation at varying levels of MgATP. B. Similar data for solubilized, purified sarcoplasmic reticulum-bound hexokinase. C. Similar data for purified hexokinase rebound to enzyme-denuded sarcoplasmic reticulum. See EXPERIMENTAL for details.

hexokinase[3] reported previously was not activated by orthophosphate under similar experimental conditions (*i.e.* 50 mM Tris buffer, pH 7.5), but was activated by orthophosphate under conditions of molecular aggregation (*i.e.* low ionic strength 15 mM Tris buffer, pH 7.5).

Rebinding of the PS hexokinase to the denuded sarcoplasmic reticulum, Fig. 4C, reestablished the biphasic orthophosphate activation characteristics of the original bound enzyme. Thus maximum orthophophate activation was again at 0.5 and 10 mM MgATP, and was approximately 48 and 56% activation respectively, see insert Fig. 4C.

Interaction of MgATP with 1,5-anhydroglucitol-6-P

The K_m of the original bound hexokinase for MgATP was 0.28 mM. Interaction with 1,5-anhydroglucitol-6-*P* (which is an analogue of glucose-6-*P* in its action as an inhibitor) appeared to be predominantly competitive* for MgATP with a K_i of 0.082 mM (concentration of inhibitor that doubles the slope), Fig. 5A. Similar values were reported previously[3] for the particulate enzyme.

Solubilization and purification of this enzyme significantly altered its kinetic

* When straight lines are extrapolated to $1/[S] = 0$, 0.5 mM 1,5-anhydroglucitol-6-*P* decreases v_{max} 27%, but increases K_m/v_{max} 756%.

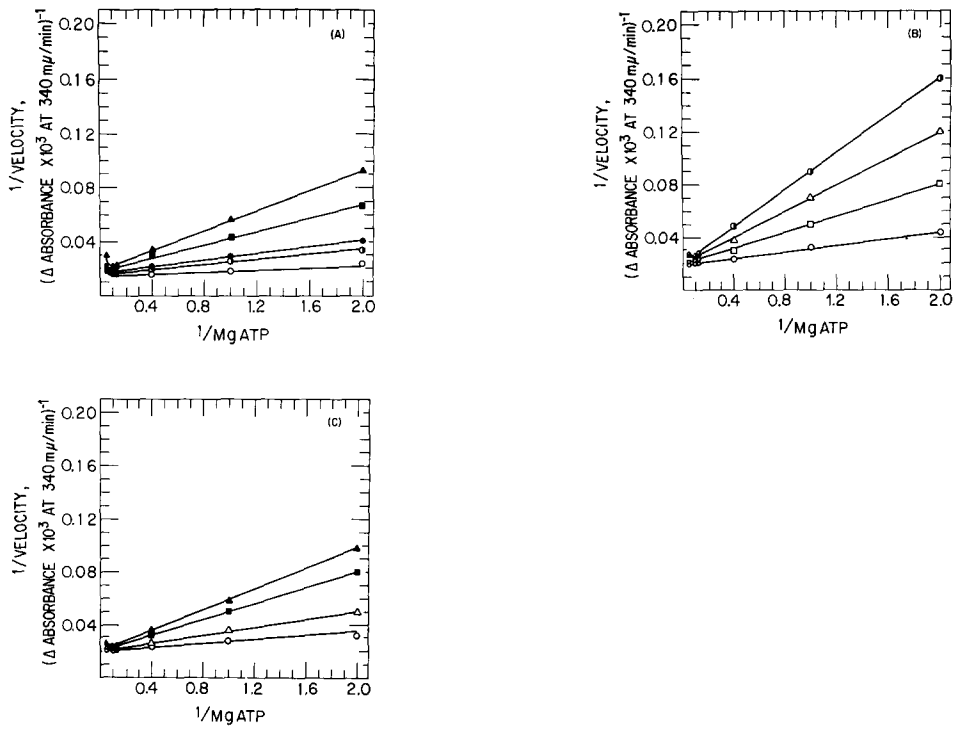


Fig. 5. A. Lineweaver–Burk plot of original sarcoplasmic reticulum-bound hexokinase for interaction of MgATP with 1,5-anhydroglucitol-6-*P*. Four levels of 1,5-anhydroglucitol-6-*P* are given: ○, 0 mM; ◑, 0.12 mM; ●, 0.2 mM; ■, 0.3 mM; and ▲, 0.5 mM. Respective MgATP K_m's for these inhibitor concentrations were: 0.28, 0.655, 0.71, 1.43 and 1.91 mM. B. Similar plot of solubilized, purified sarcoplasmic reticulum-bound hexokinase. Three levels of 1,5-anhydroglucitol-6-*P* are given: ○, 0 mM; □, 0.04 mM; △, 0.08 mM; ◑, 0.12 mM. Respective MgATP K_m's for these inhibitor concentrations were: 0.64, 1.54, 2.57 and 3.59 mM. C. Similar plot for solubilized, purified hexokinase rebound to enzyme-denuded sarcoplasmic reticulum. Three levels of 1,5-anhydroglucitol-6-*P* are given: ○, 0 mM; △, 0.08 mM; ■, 0.3 mM; and ▲, 0.5 mM. MgATP K_m's for these inhibitor concentrations were: 0.42, 0.77, 1.47, 2.00 mM.

properties by raising the sensitivity to 1,5-anhydroglucitol-6-*P*, 2.1-fold (lowered K_i to 0.026 mM). The K_m for MgATP was raised slightly to 0.64 mM, Fig. 5B. Thus 0.08 mM 1,5-anhydroglucitol-6-*P* raised the K_m for MgATP 1.3-fold for the more resistant particulate enzyme, whereas the same concentration of inhibitor raised the K_m 4-fold for the solubilized particulate hexokinase (PS). It should be recalled that increased sensitivity to 1,5-anhydroglucitol-6-*P* was also noted for the OS purified hexokinase[3].

Rebinding of PS hexokinase to sarcoplasmic reticulum again restored the properties of the original bound hexokinase. The resistance to 1,5-anhydroglucitol-6-*P* inhibition was reestablished and the K_m for MgATP slightly lowered. The K_i for 1,5-anhydroglucitol-6-*P* became 0.11 mM and the K_m for MgATP, 0.41 mM, Fig. 5C.

Binding and solubilization of sarcoplasmic reticulum-bound hexokinase

The binding affinity of hexokinase for sarcoplasmic reticulum was greatest at

TABLE II

EFFECT OF pH ON REPEATED SOLUBILIZATION OF ORIGINAL SARCOPLASMIC RETICULUM-BOUND HEXOKINASE

Data represent average of 4 experiments performed in the presence of 5 mM EDTA, 5 mM mercaptoethanol, 50 mM Tris buffer.

	pH 6.5		*pH 7.5*		*pH 8.5*	
	*% Solubilization***	*% Recovery****	*% Solubilization*	*% Recovery*	*% Solubilization*	*% Recovery*
Original pellet*	—	100	—	100	—	100
S_1P_1	43	112	55	114	49	98
S_2P_2	9	94	49	101	10	98
S_3P_3	5	90	60	120	8	101
S_4P_4	6	71	53	93	4	100

* Original sarcoplasmic reticulum-bound hexokinase.

** Refers to percent hexokinase activity solubilized from the particulate fraction (total activity) from which it was derived.

*** Refers to % total activity recovered ($P_n + S_n$), from the previous particulate fraction (P_{n-1}), *i.e.* $[(P_n + S_n)/P_{n-1}] \times 100$.

pH 6.5 and 8.5, Table II. The dissociation constant K_D was calculated from the average % solubilized enzyme of S2, S3, and S4. Thus the K_D at pH 6.5, 8.5 and 7.5 was 0.48, 0.54 and 63.4 units/ml, respectively. Thus the binding affinity ($1/K_D$) was much greater at pH 6.5 and 8.5.

DISCUSSION

These data provide evidence for the cellular organization of a regulator enzyme, hexokinase. They clearly indicate the danger of overinterpreting the kinetics of a crystalline purified enzyme when removed from its cellular environment. Binding of hexokinase to sarcoplasmic reticulum results in activation by orthophosphate at low ATP concentrations, whereas orthosphosphate activation of the unbound hexokinase is operative at high ATP concentration only. Binding of hexokinase to sarcoplasmic reticulum results in 3 fold greater resistance to 1,5-anhydroglucitol-6-*P* (with respect to MgATP) at physiologic concentration of substrate and inhibitor. Thus binding of this enzyme to cell components significantly alters its kinetic properties.

In frog skeletal muscle, particulate hexokinase represents at least 50% of total hexokinase activity[2] and is distributed between the mitochondrial and sarcoplasmic reticulum fraction. The latter represents at least 25% of the total skeletal muscle hexokinase activity[2]. Similar binding of hexokinase to microsomal fractions has been reported in guinea pig cerebral cortex[8], bovine adrenal medulla[9] and rat epididymal adipose tissue[10].

The alteration of kinetic properties of the sarcoplasmic reticulum-bound and unbound hexokinase of frog skeletal muscle contributes to the hypothesis of the cellular organization of this regulator enzyme. The bound enzyme appears to be the physiologically more active hexokinase with a lower K_m for MgATP, a greater resistance to glucose-6-*P* inhibition, and an optimum activation by orthophosphate at physiologic MgATP concentration. It is of interest, in this regard, that particulate

hexokinases from other tissues have also been shown to have a significantly lower K_m for MgATP and greater resistance to glucose-6-*P* inhibition, when compared to their respective soluble enzymes[11–16]. The association of hexokinase with sarcoplasmic reticulum in frog skeletal muscle lends further support to such a regulatory mechanism. The sarcoplasmic reticulum is believed to act as a calcium pump which serves to regulate the contraction-relaxation phase of muscle[17,18].

The orthophosphate activation kinetics of frog skeletal muscle hexokinase appears to be unique for this enzyme. Orthophosphate, *per se*, will activate the enzyme in the absence as well as presence of inhibitor (1,5-anhydroglucitol-6-*P*)[2]. The present report further delineates this activation process, which has been shown to be dependent upon MgATP concentration as well as the bound state of the enzyme. At varying MgATP concentration, optimum orthophosphate activation is biphasic for the bound enzyme and monophasic for the purified solubilized enzyme.

It is intriguing to compare the solubilized purified particulate hexokinase (PS)

TABLE III

COMPARISON OF ORIGINAL SOLUBLE HEXOKINASE WITH SOLUBILIZED PARTICULATE HEXOKINASE

	Original soluble enzyme purified 180-fold	*Particulate sarcoplasmic reticulum-bound enzyme*	*Solubilized particulate enzyme purified 60-fold*
Molecular weight			
Sephadex G-200	105 000		112 875
Sucrose gradient	99 700		95 212
Molecular Aggregation characteristics at 2–3 mg/ml (average)	15%		50–100%
Glucose K_m	0.1 mM	0.09 mM	0.09 mM
ATP K_m	1.48 mM	0.28 mM	0.64 mM
1,5-Anhydroglucitol-6-*P* K_i for MgATP	0.026 mM	0.08–0.11 mM	0.026 mM
Maximum activation by P_i	0	Biphasic at 0.5 and 10 mM MgATP	10 mM MgATP
Binding affinity for sarcoplasmic reticulum (average bound)	6%		40%

with the original 105 000 × *g* soluble hexokinase (OS) of frog skeletal muscle[3], Table III. The molecular weight, K_m for glucose, K_m for MgATP and K_i for 1,5-anhydroglucitol-6-*P* inhibition of MgATP appear to be the same. However, the propensity for molecular aggregation is considerably greater for PS hexokinase compared to OS hexokinase. Likewise, activation by orthophosphate is retained by PS hexokinase following solubilization, but only at high ATP concentration; whereas OS hexokinase is not activated by orthophosphate in 50 mM Tris buffer, pH 7.5.

However, following aggregation in low ionic strength buffer, OS hexokinase is activated by orthophosphate, particularly at high ATP concentration[3]. These data indicate close similarities between the OS and PS hexokinase of frog skeletal muscle. The PS hexokinase appears to have a site or conformation which contributes to its enhanced molecular aggregation, binding affinity and orthophosphate activation.

It is conceivable that all of the hexokinase of frog skeletal muscle is particulate bound. Homogenization and extraction with release of lysosomal enzymes could lead to significant alterations of the OS hexokinase with destruction or alteration of the binding site of the enzyme. This is substantiated to some extent by the diminished binding properties of the purified OS hexokinase to denuded sarcoplasmic reticulum (approximately 6% of the enzyme can be bound compared to 40% for the PS hexokinase (S. KARPATKIN AND J. BRAUN, unpublished observations.).

An alternative explanation is that these two enzymes are closely related isoenzymes.

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