Interaction between Muscle Aldolase and Muscle Fructose 1,6-Bisphosphatase Results in the Substrate Channeling†

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ABSTRACT: Fructose 1,6-bisphosphatase (FBPase) is known to form a supramolecular complex with α-actinin and aldolase on both sides of the Z-line in skeletal muscle cells. It has been proposed that association of aldolase with FBPase not only desensitizes muscle FBPase toward AMP inhibition but it also might enable the channeling of intermediates between the enzymes [Rakus et al. (2003) *FEBS Lett. 547*, 11−14]. In the present paper, we tested the possibility of fructose 1,6-bisphosphate (F1,6-P_2) channeling between aldolase and FBPase using the approach in which an inactive form of FBPase competed with active FBPase for binding to aldolase and thus decreased the rate of aldolase−FBPase reaction. The results showed that F1,6-P_2 is transferred directly from aldolase to FBPase without mixing with the bulk phase. Further evidence that F1,6-P_2 is channeled from aldolase to FBPase comes from the experiments investigating the inhibitory effect of a high concentration of magnesium ions on aldolase−FBPase activity. FBPase in a complex with aldolase, contrary to free muscle FBPase, was not inhibited by high Mg^{2+} concentrations, which suggests that free F1,6-P_2 was not present in the assay mixture during the reaction. A real-time interaction analysis between aldolase and FBPase revealed a dual role of Mg^{2+} in the regulation of the aldolase−FBPase complex stability. A physiological concentration of Mg^{2+} increased the affinity of muscle FBPase to muscle aldolase, whereas higher concentrations of the cation decreased the concentration of the complex. We hypothesized that the presence of Mg^{2+} stabilizes a positively charged cavity within FBPase and that it might enable an interaction with aldolase. Because magnesium decreased the binding constant (K_a) between aldolase and FBPase in a manner similar to the decrease of K_a caused by monovalent cations, it is postulated that electrostatic attraction might be a driving force for the complex formation. It is presumed that the biological relevance of F1,6-P_2 channeling between aldolase and FBPase is protection of this glyconeogenic, as well as glycolytic, intermediate against degradation by cytosolic aldolase, which is one of the most abundant enzyme of glycolysis.

A key enzyme of glyconeogenesis is fructose 1,6-bisphosphatase (FBPase,[1] EC 3.1.3.11), which catalyzes hydrolysis of fructose 1,6-bisphosphate (F1,6-P_2) to fructose 6-phosphate (F6-P) and inorganic phosphate in the presence of divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}) (*1*, *2*). Both mammalian FBPase isozymes, liver and muscle, are activated by monovalent cations (*1*−*3*) and inhibited competitively by fructose 2,6-bisphosphate (F2,6-P_2) and allosterically by AMP (*1*−*6*). Mammalian FBPase is a homotetramer (subunit mass of 37 000 Da) (*7*−*10*) and exists in at least two quaternary conformations called R and T depending on the relative concentrations of the enzyme effectors (*6*, *11*). A proposed mechanism for allosteric regulation of catalysis involves three conformational states of loop 52−72 called engaged, disengaged, and disordered (*12*). AMP stabilizes a disengaged loop (*13*−*14*), whereas metals with products stabilize an engaged loop (*14*−*16*). The enzyme is active if loop 52−72 can cycle between its engaged and disordered conformations (*12*−*15*).

Muscle FBPase isozyme exists both as a soluble constituent of the cell and as the enzyme associated with α actinin of the Z line (*17*, *18*). It is believed that a sarcomere-bound form of FBPase interacting with aldolase (*19*, *20*) and other glyconeogenic enzymes form a glyconeogenic complex within the region of the Z line (*8*). Although the muscle isozyme of aldolase (aldolase A) is usually regarded as an enzyme especially tailored for glycolysis, it can also catalyze synthesis of F1,6-P_2 from triose phosphates (*21*). This way aldolase A may participate not only in glycolysis but also in the synthesis of glycogen from carbohydrate precursors, which has been observed in skeletal muscles (*22*−*24*).

The interaction between aldolase and FBPase, which is affected by the metabolic state of the cell, seems to be a regulatory point of glycogen synthesis from carbohydrate

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[1] Abbreviations: FBPase, fructose 1,6-bisphosphatase; F1,6-P_2, fructose 1,6-bisphosphate; F6-P, fructose 6-phosphate; F2,6-P_2, fructose 2,6-bisphosphate; PEG 8000, poly(ethylene glycol) 8000; G6PDH, glucose 6-phosphate dehydrogenase; PGI, glucose 6-phosphate isomerase; TIM, triose 3-phosphate isomerase; DHAP, dihydroxyacetone phosphate; OPTA, *o*-phthalaldehyde; PLP, pyridoxal 5′-phosphate.

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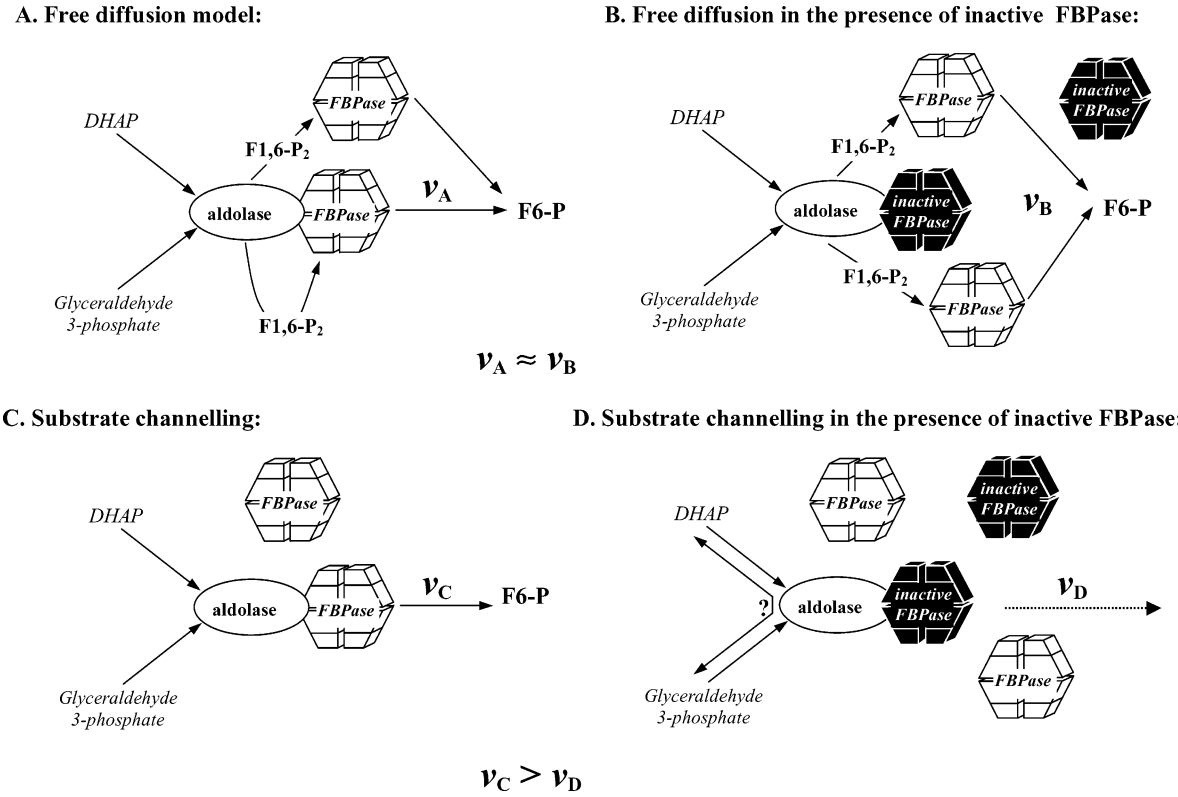


FIGURE 1: Models describing the transfer of F1,6-P_2 from aldolase to FBPase. In the presence of a large excess of FBPase over aldolase (molar ratio ~ 100), the production of F1,6-P_2 is practically catalyzed only by aldolase bound with FBPase. In the free diffusion model (A and B), if FBPase does not change the catalytic properties of aldolase, the rate of F6-P synthesis is almost not affected by the presence of inactive, OPTA-labeled FBPase (B). If the substrate channeling occurs (C and D), the velocity of the reaction decreases upon the addition of the inactive form of FBPase (D).

precursors in myocytes (*20*). The activity of the free form of muscle FBPase is completely inhibited by the physiological concentration of AMP (*25*). The interaction between muscle aldolase and muscle FBPase entirely desensitizes the latter enzyme to AMP inhibition, enabling glycogen synthesis from carbohydrate precursors (*19*, *20*). Because the stability of the FBPase−aldolase complex is down-regulated by the intermediates, which bind to the active sites of the enzymes, channeling or restricted free-diffusion mechanism of F1,6-P_2 transfer from aldolase to FBPase was postulated (*20*).

Substrate channeling is a process in which the intermediate produced by one enzyme is directly transferred to the next enzyme without complete equilibration with the bulk phase. The advantages of channeling are numerous: isolation of intermediates from competing reactions, decrease of transit times of intermediates, prevention from the loss of intermediates by diffusion, protection of labile intermediates from the solvent, etc. (for review, see refs *26*−*28*).

In the recent years, it has been demonstrated that sequential enzymes that operate within such metabolic pathways as the tricarboxylic acid cycle (*29*), urea cycle (*30*), ribosomal protein synthesis (*31*), and glycolysis (*32*) interact with each other to form highly organized complexes facilitating substrate channeling. Much data suggests that the phenomenon of substrate channeling occurs between at least two enzymes of the glycolytic pathway (aldolase and glyceraldehyde 3-phosphate dehydrogenase) (*33*, *34*) and that substrate channeling may be crucial for the regulation of glycolysis in skeletal muscle cells (for review, see refs *27* and *32*). Considerably less information is available on the spatial and functional organization of muscle glyconeogenesis.

To investigate the hypothesis that substrate channeling occurs between muscle aldolase and muscle FBPase, we developed a general methodology of testing substrate channeling, previously described by Geck and Kirsch (*35*) (Figure 1). This approach makes it possible to establish the existence (or nonexistence) of direct substrate transfer between consecutive enzymes in a metabolic pathway.

The results presented here provide direct kinetic evidence for the substrate channeling mechanism in myofibrillar glyconeogenesis. It is also presumed that substrate channeling between aldolase and FBPase, precluding diffusion of the intermediate into the bulk phase and thereby protecting F1,6-P_2 against its degradation by glycolytic pathway, may be regarded as an elementary mechanism enabling glycogen synthesis from carbohydrate precursors in myocytes.

EXPERIMENTAL PROCEDURES

Materials. Phosphocellulose P-11 was purchased from Whatman (Maidstone, U.K.), ethanolamine was from Merck (Germany), and BIAcore sensor chip CM5 was from Biacore AB (Uppsala, Sweden). Other reagents were from Sigma (St. Louis, MO).

Enzyme Purification and the Activity Determination. Rabbit muscle aldolase and FBPase were purified according to Penhoet et al. (*21*) and Rakus et al. (*36*), respectively. The activities of aldolase and FBPase were determined as described by Gracy et al. (modified) (*37*) and Rakus and Dzugaj (*38*), respectively.

The assay for measurement of the FBPase−aldolase complex activity was described previously (*20*) and, with slight modifications, was employed in the present studies. The standard assay medium contained 20 mM Tris, 0.25 mM EDTA, 1.25 mM $MgCl_2$, 50 mM KCl, 10% poly(ethylene glycol) (PEG 8000), 0.5 mM NADP, 50 units/mL of glucose 6-phosphate dehydrogenase (G6PDH), 50 units/mL of glucose 6-phosphate isomerase (PGI), 50 units/mL of triose 3-phosphate isomerase (TIM), and 0.08 mM dihydroxyacetone phosphate (DHAP) at pH 7.5 and $T = 37$ °C. Unless otherwise specified, 0.1 μg/mL of aldolase (0.625 nM tetramer concentration) and 10 μg/mL of FBPase (68 nM concentration of the tetramer) were used throughout all experiments. The effect of K^+, Na^+, and Mg^{2+} on the rate of the reaction catalyzed by the aldolase−FBPase complex was determined in the presence of 5 μM AMP to ensure that only the activity of the complex was measured; muscle FBPase unbound to aldolase is almost completely inhibited by such AMP concentrations (*9*, *19*, *25*). On the other hand, 5 μM AMP has no effect on the complex activity or stability (*19*, *20*).

One unit of an enzyme activity is defined as the amount of the enzyme that catalyzes the formation of 1 μmol of product per minute.

Spectrophotometric measurements were performed with a Agilent 8453 diode array spectrophotometer. All kinetic calculations were performed using Microsoft Excel 2000 and GraFit 4 programs (*39*).

Aldolase and FBPase Labeling. Modifications of the active site of rabbit muscle aldolase were performed with *o*-phthalaldehyde (OPTA) (*40*) and with pyridoxal 5′-phosphate (PLP) (*41*).

To obtain the inactive form of muscle FBPase, the enzyme was labeled with OPTA as described by Dzugaj et al. (*42*) and Puri and Roskoski (*43*). After the labeling, the enzyme was extensively dialyzed against 20 mM TRIS and 0.05 mM EDTA at pH 7.5 and $T = 4$ °C.

The lack of proteolysis of the labeled proteins was checked by 10% SDS−PAGE (*44*).

Real-Time Interaction Analysis by BIAcore. The binding of various forms of aldolase to various forms of FBPase was measured using BIAcore 1000 (Biacore AB, Uppsala, Sweden) as described by Rakus et al. (*18*, *20*) in the absence of KCl.

To check the effect of Mg^{2+}, Na^+, and K^+ on the affinity of aldolase to FBPase, under conditions imitating the physiological, the binding medium was supplemented with 7% PEG 8000 as a crowding agent (*45*).

Test for Substrate Channeling. The following method, described earlier by Geck and Kirsch (*35*) was developed to check the existence of substrate channeling between aldolase and FBPase (Figure 1).

The activity of the aldolase−FBPase complex was measured in the assay mixture containing 0.1 μg/mL (0.625 nM) of aldolase, 10 μg/mL (68 nM) of FBPase, 20 mM Tris, 0.25 mM EDTA, 1.25 mM $MgCl_2$, 50 mM KCl, 10% PEG 8000, 0.5 mM NADP, 50 units/mL of G6PDH, 50 units/mL of PGI, 50 units/mL of TIM, and 0.08 mM DHAP at pH 7.5 and $T = 37$ °C. Several assays were performed in which the concentrations of aldolase and FBPase were held constant, and increasing concentrations of inactive OPTA-labeled FBPase were added.

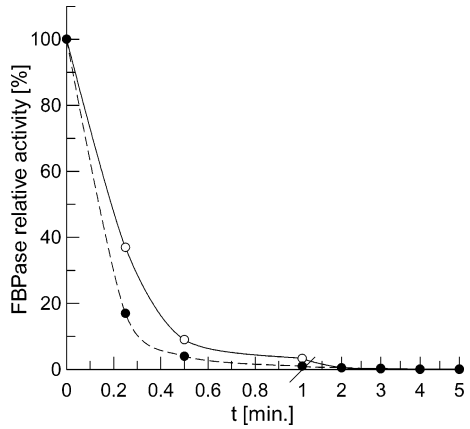


FIGURE 2: Time-dependent heat denaturation of rabbit muscle FBPase (—, ○) and rabbit muscle aldolase (- - -, ●). The samples of FBPase (10 μg/mL, ~34 nM tetramer concentration) and aldolase (10 μg/mL, ~0.625 nM concentration of the tetramer) were incubated at various times at 90 °C in a buffer containing 10% PEG 8000, 20 mM TRIS, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 20 units/mL of TIM, and 80 μM DHAP at pH 7.5 and $T = 37$ °C, and the activities of aldolase and FBPase were determined as described by Gracy et al. (modified) (*37*) and Rakus and Dzugaj (*38*), respectively.

Because the test for channeling requires that aldolase, upon binding to inactive FBPase, should be at least partially active, the rate of F1,6-P_2 synthesis and its release into the bulk phase by aldolase, in the absence as well as in the presence of 10 μg/mL (68 nM) of native FBPase or 20 μg/mL (136 nM) of OPTA-modified FBPase, was checked. A total of 0.1 μg/mL (0.625 nM) of aldolase was incubated in the mixture containing 10% PEG 8000, 20 mM TRIS, 50 mM KCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 20 units of TIM, and 80 μM DHAP at pH 7.5 and $T = 37$ °C, in the absence and presence of various forms of FBPase. To protect F1,6-P_2 from degradation by free native FBPase, 5 μM AMP was added to the reactions. This amount of the inhibitor completely inhibits the activity of muscle FBPase (*9*, *25*). Upon 0, 5, 10, 15, and 20 min of the incubation, the sample was denatured at 90 °C for 5 min (time-dependent heat denaturation of free aldolase and free FBPase is demonstrated in Figure 2) and centrifuged (20 min, $T = 4$ °C, 20000*g*), and the amount of F1,6-P_2 was determined spectrophotometrically as follows: the samples (500 μL) were preincubated with 500 μL of mixture containing 20 mM TRIS, 0.25 mM EDTA, 1.25 mM $MgCl_2$, 400 μM NADH, 20 units/mL TIM, and 40 units/mL of glycerophosphate dehydrogenase at pH 7.5 and $T = 37$ °C until the baseline was obtained, and aldolase was added to start the reaction of F1,6-P_2 cleavage. The concentration of F1,6-P_2 in the reaction mixture was also checked under conditions disabling the complex formation (the assay was depleted of the crowding agent, PEG 8000).

The set of parallel reactions, in which the amount of F6-P synthesized by various forms of FBPase was measured, were also performed. The mixtures for determination of F6-P concentrations contained 20 mM TRIS, 0.25 mM EDTA, 1.25 mM $MgCl_2$, 0.5 mM NADP, 20 units/mL of G6PDH, and 20 units/mL of PGI at pH 7.5 and $T = 37$ °C, and the changes of NADPH absorbance were monitored spectrophotometrically.

Table 1: Kinetic Data on the Interaction of Various Forms of Muscle Aldolase with Various Forms of Muscle FBPase, as Measured by Surface Plasmon Resonance

complex	K_a[a] (1/M)
FBPase Immobilized on the Surface of the Sensor Chip	
aldolase−FBPase	2.1×10^8 (2.8×10^6)
$aldolase_{OPTA}$−FBPase	9.6×10^4 (5.8×10^2)
$aldolase_{PLP}$−FBPase	3.3×10^4 (2.1×10^3)
Aldolase Immobilized on the Surface of the Sensor Chip	
FBPase−aldolase	1.3×10^8 (3.7×10^6)
$FBPase_{OPTA}$−aldolase	6.7×10^7 (7.4×10^5)

[a] Each K_a value represents the mean and SE of triplicate determinations.

RESULTS

Interactions between Various Forms of FBPase and Various Forms of Aldolase. The interaction between FBPase and aldolase was drastically affected by the modification of the latter enzyme with its active-site-specific reagents, OPTA [modification of Lys 229 and Cys 149 (*40*)] and PLP [Lys 107 is supposed to be labeled in this case (*41*)]. Muscle FBPase bound OPTA-modified aldolase ($aldolase_{OPTA}$) and PLP-labeled aldolase ($aldolase_{PLP}$) respectively about 2000 and 6000 times more weakly than the native muscle aldolase (Table 1). Although OPTA and PLP modify amino acids residues within the aldolase structure, which are directly involved in the substrates binding and catalysis, both modified aldolases were partially active (data not shown).

The interaction between the OPTA-labeled form of muscle FBPase ($FBPase_{OPTA}$) and aldolase was 2-fold weaker than the binding of native muscle FBPase to aldolase (Table 1). Because $FBPase_{OPTA}$ was almost completely inactive (specific activity was less than 10 milliunits/mg), this form of the enzyme was suitable to test the possibility of substrate channeling between aldolase and FBPase.

Effect of Various Forms of FBPase on the Activity of Aldolase. Theoretically, the decrease of F6-P synthesis in the test for channeling upon addition of an inactive form of FBPase may be caused by blocking the substrate channeling as well as by inhibition of aldolase activity. Therefore, the test requires that the inactive form of FBPase should not inhibit the activity of aldolase completely.

The results presented in Figure 3 demonstrate that $FBPase_{OPTA}$ only slightly inhibited (~20%) the activity of aldolase. On the other hand, the hydrolysis of F1,6-P_2 to F6-P was observed only when native FBPase was associated with aldolase. In this case, the presence of F1,6-P_2 was almost undetectable in the reaction mixture.

Effect of Monovalent Cations on the Stability and on the Activity of the Aldolase−FBPase Complex. Certain monovalent cations (such as K^+, NH_4^+, or Tl^+) are required for FBPase to achieve its maximal catalytic activity (*1*−*3*). Surprisingly, the rate of the reaction catalyzed by the aldolase−FBPase complex was inhibited by KCl, with $I_{0.5}$ (concentration of the effector that inhibits an enzyme activity 2-fold) equal to 107 mM (Figure 4). Almost the same value of $I_{0.5}$ (95 mM) was obtained when the effect of Na^+ on the complex activity was tested (Figure 4), although sodium ions are not believed to significantly affect FBPase catalysis (*46*). Presumably, the inhibitory effect of Na^+ as well as K^+ on the complex activity resulted only from the decreased concentration of the aldolase−FBPase complex caused by ionic strength. BIAcore-based analysis showed that potassium and sodium cations strongly decrease the affinity of FBPase to aldolase (Figure 4 and parts a and b of Figure 5).

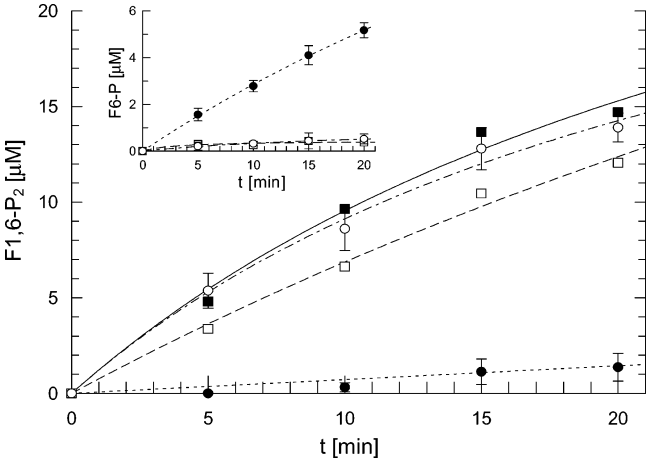


FIGURE 3: Changes in the concentration of F1,6-P_2 synthesized by aldolase determined in the absence of FBPase (—, ■), as well as in the presence of muscle FBP_{OPTA} (- - -, □) and native muscle FBPase (···, ●) under conditions enabling aldolase−FBPase complex formation. (-·-, ○) Rate of F1,6-P_2 synthesis under conditions disabling aldolase−FBPase complex formation. Inset shows the changes in the concentration of F6-P as determined in the parallel reactions (the changes of the F6-P concentration in the absence of FBPase were not detectable, and thus, they are not demonstrated). The effect of native FBPase on F1,6-P_2 synthesis by aldolase was determined in the presence of 5 μM AMP, which completely inhibits muscle FBPase. Every data point is the mean ± standard deviation (SD) of three independent metabolite concentration measurements.

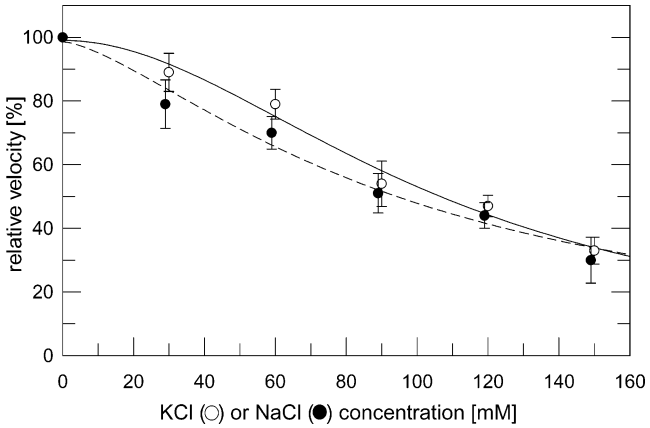


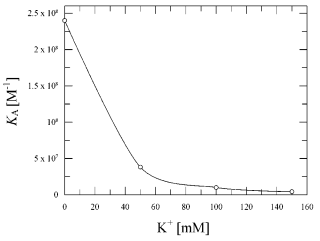
FIGURE 4: Effect of K^+ (—, ○) and Na^+ (- - -, ●) on the rate of F6-P synthesis by the aldolase−FBPase complex. The reaction mixture contained 0.1 μg/mL (0.625 nM) of aldolase and 10 μg/mL (68 nM) of FBPase. Every data point is the mean ± SD of three independent enzymatic activity determinations.

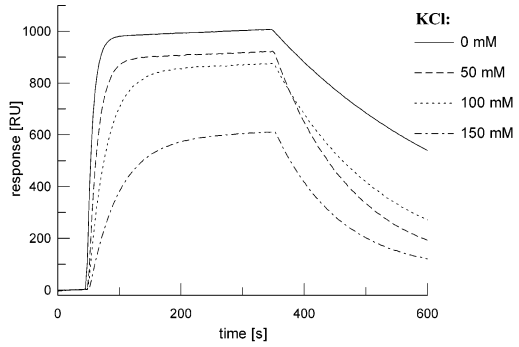
Stability and the Activity of Aldolase−FBPase Complex in the Presence of Various Concentrations of Magnesium Ions. The stability of the aldolase−FBPase complex *in vitro* is regulated by magnesium ions (Figure 5c). Physiological concentrations (1−2 mM) of Mg^{2+} increased the binding constant (K_a) about 10 times compared to the K_a determined in the absence of Mg^{2+}. However, the higher concentrations of the divalent cations decreased the stability of the complex, presumably increasing the ionic strength of the binding solution.

Mg^{2+} cooperatively activates animal FBPases with a Hill coefficient (n) of approximately 2 (*1*, *2*). The concentration

a)

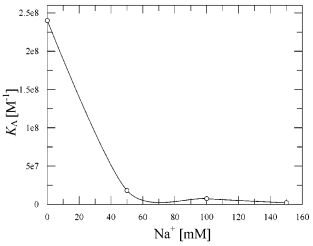
effector	K_A [1/M]
K^+ – 0 mM Mg^{2+} – 1 mM	**2.4 x 10^8** SE 1.1 x 10^6
K^+ – 50 mM Mg^{2+} – 1 mM	**3.8 x 10^7** SE 4.9 x 10^5
K^+ – 100 mM Mg^{2+} – 1 mM	**1.0 x 10^7** SE 2.7 x 10^5
K^+ – 150 mM Mg^{2+} – 1 mM	**4.2 x 10^6** SE 6.8 x 10^4

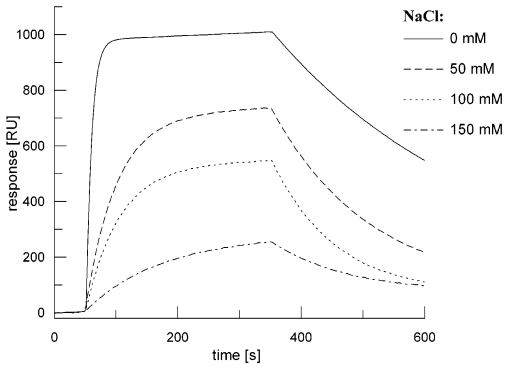




b)

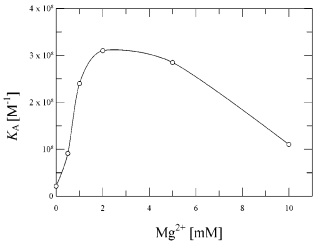
effector	K_A [1/M]
Na^+ – 0 mM Mg^{2+} – 1 mM	**2.4 x 10^8** SE 1.1 x 10^6
Na^+ – 50 mM Mg^{2+} – 1 mM	**1.8 x 10^7** SE 5.3 x 10^5
Na^+ – 100 mM Mg^{2+} – 1 mM	**7.4 x 10^6** SE 3.8 x 10^5
Na^+ – 150 mM Mg^{2+} – 1 mM	**2.0 x 10^6** SE 8.7 x 10^4





c)

effector	K_A [1/M]
Mg^{2+} – 0	**2.1 x 10^7** SE 3.1 x 10^5
Mg^{2+} – 0.5 mM	**9.1 x 10^7** SE 5.3 x 10^5
Mg^{2+} – 1 mM	**2.4 x 10^8** SE 1.1 x 10^6
Mg^{2+} – 2 mM	**3.1 x 10^8** SE 4.9 x 10^6
Mg^{2+} – 5 mM	**2.9 x 10^8** SE 1.7 x 10^6
Mg^{2+} – 10 mM	**1.1 x 10^8** SE 1.9 x 10^6



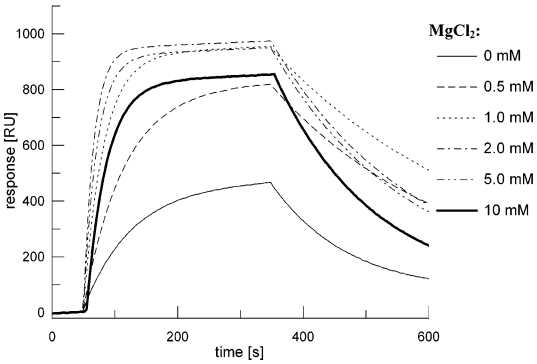


FIGURE 5: Kinetic data on the binding of muscle FBPase to muscle aldolase immobilized on the surface of Biacore biosensor in the presence of 7% PEG 8000, as measured by surface plasmon resonance. (a) Effect of K^+ on the affinity of FBPase (156 nM concentration of monomer) to aldolase. (b) Effect of Na^+ on the affinity of FBPase (156 nM concentration of monomers) to aldolase (c) Effect of Mg^{2+} on the affinity of FBPase (39 nM concentration of monomers) to aldolase. The dependence of K_a on the cations concentration is presented in the tables and, for clarity, also graphs. The Bezier curve was used to join the experimental points. Each K_a value represents the mean and standard error (SE) of triplicate determinations. Representative sensograms for each investigated concentration of cations are demonstrated.

of Mg^{2+} required to activate muscle FBPase to its half-maximal activity ($A_{0.5}$) was estimated to be 0.16 mM (Figure 6). The activation of the FBPase−aldolase complex was much stronger ($A_{0.5}$ was 0.036 mM), and the cooperativity was lost (Figure 6). The observed lack of cooperativity seems to be apparent and may be explained assuming that Mg^{2+} not only activates FBPase but also enhances its affinity to aldolase. An overlap of these two simultaneous phenomena should result in an apparent loss of cooperativity and in an apparent increase of the sensitivity toward the activator (under the experimental conditions, the velocity of F6-P synthesis depends on the concentration of the aldolase−FBPase complex, in which FBPase is associated with Mg^{2+}). High Mg^{2+} concentrations inhibit free FBPase activity

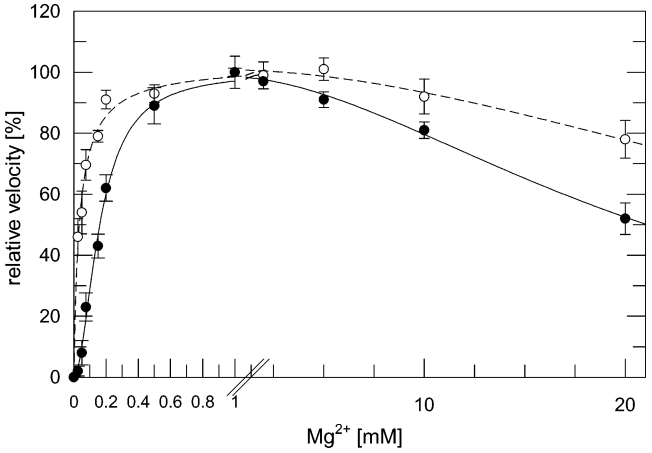


FIGURE 6: Effect of Mg^{2+} on the activity of the aldolase−FBPase complex. Dependence of the activity of the FBPase−aldolase complex on the concentration of Mg^{2+} in the absence of potassium ions (- - -, ○). The effect of Mg^{2+} on the activity of FBPase in the absence of aldolase is also shown (—, ●). Every data point is the mean ± SD of three independent enzymatic activity determinations.

complexing and decreasing the concentration of the enzyme substrate, F1,6-P_2 (*47*). Therefore, if aldolase released F1,6-P_2 into the bulk phase, free FBPase and aldolase-associated FBPase should be inhibited by Mg^{2+} in the same manner. Data presented in Figure 6 shows that FBPase in the presence of aldolase was significantly more weakly inhibited by high magnesium concentrations (∼80% of the maximal velocity) than in the absence of aldolase (∼50% of the maximal velocity). Such results strongly suggest that the inhibition of the complex could not be an effect of chelating of FBPase substrate by Mg^{2+}. Moreover, there is evidence that the inhibition of the complex by high magnesium concentrations was probably caused only by an increase of the ionic strength of the solution, which results in the decrease of the complex concentration (Figure 5c). Thus, if FBPase associated with aldolase was not, in practice, inhibited by high Mg^{2+} concentrations, it means that F1,6-P_2 could not diffuse away into free solution during the complex reaction.

Effect of Various Concentrations of FBPase on the Rate of F6-P Synthesis in the Presence and Absence of PEG 8000. The activity of the complex was constant and independent of FBPase concentrations higher than 5 μg/mL (34 nM tetramer concentration) (Figure 7a). When the rate of F6-P synthesis was measured under conditions precluding the complex formation (the reaction buffer contained 150 mM KCl and was depleted of PEG 8000), the increasing concentrations of FBPase, up to 200 μg/mL (1.36 μM concentration of the tetramer, ∼7.5 units/mL), resulted in the increase of the reaction rate (Figure 7a). The maximal activity of the unbound aldolase was about 4 times higher than the velocity of aldolase in a complex with FBPase (Figure 7a). FBPase may play a dual role with respect to aldolase. First, FBPase may form a complex with aldolase (*19*, *20*); second, FBPase may work as one of the coupling enzymes in the assay for aldolase activity measurement (*20*). In the second case, the concentration of FBPase in the reaction mixture must be manyfold higher than in the first. It is evident that 5 μg/mL (34 nM, ∼180 milliunits/mL) of FBPase is not sufficient to complete the general requirements for the coupling enzyme (see ref *48*). Experimental data

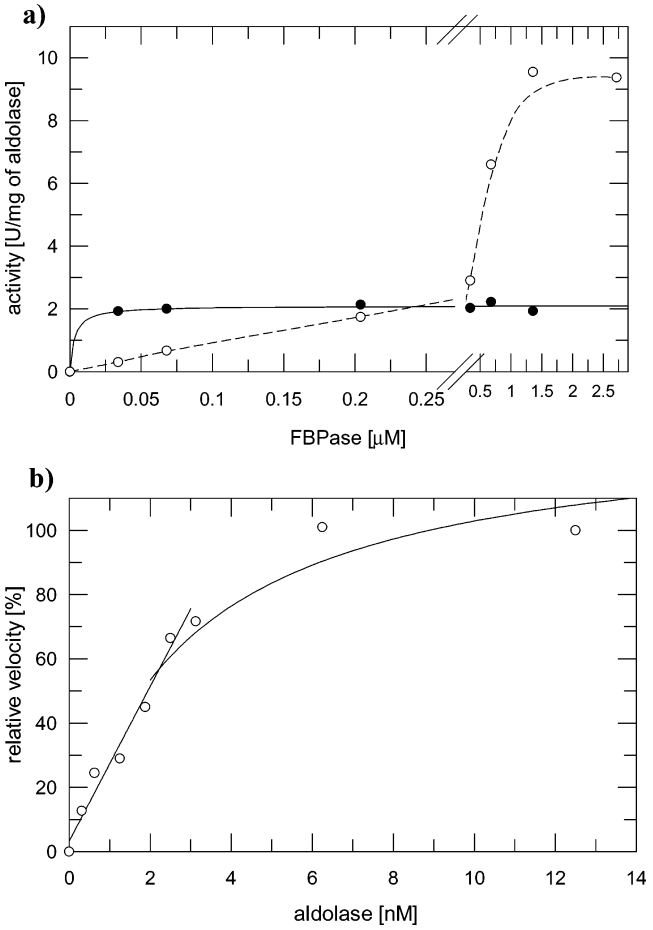


FIGURE 7: (a) Effect of FBPase on the rate of F6-P synthesis by the aldolase−FBPase coupled system. The velocity of the aldolase−FBPase complex is independent of over 5 μg/mL (34 nM) FBPase added to the reaction (—, ●). Under conditions protective to the complex formation, an increase in the FBPase concentration causes the increase of the rate of F6-P synthesis by aldolase (- - -, ○). (b) Effect of aldolase on the rate of F6-P synthesis by the aldolase−FBPase coupled system. In the presence of 10 μg/mL (68 nM) of FBPase, the linear dependence of F6-P synthesis on aldolase concentration is observed for aldolase concentrations within the range of 0−0.5 μg/mL (0−3.125 nM). It is assumed that, within this range, only the activity of the complex but not of free aldolase is measured.

(Figure 7a) and performed calculations show that at least 200 μg/mL (1.36 μM, ∼7.5 units/mL) of FBPase must be used to fulfill the requirements for the coupling enzyme in the assay for aldolase activity measurement. On the other hand, 5 μg/mL (34 nM) of FBPase almost completely saturates 0.1 μg/mL (0.625 nM) of aldolase under conditions enabling the complex formation (Figure 5a).

The reactions were carried out in the absence of AMP.

Effect of Various Concentrations of Aldolase on the Rate of F6-P Synthesis in the Presence of PEG 8000. The experimental rationale for the test for channeling requires that the velocity of the complex must depend only on the aldolase concentration. The data presented in Figure 7b shows that the rate of F6-P synthesis by the aldolase−FBPase complex in the presence of 10 μg/mL (58 nM) of FBPase was linear up to 0.5 μg/mL (3.125 nM) of aldolase added to the reaction (Figure 7b). Thus, the concentration of aldolase (0.1 μg/mL) within this range was employed in the test for channeling.

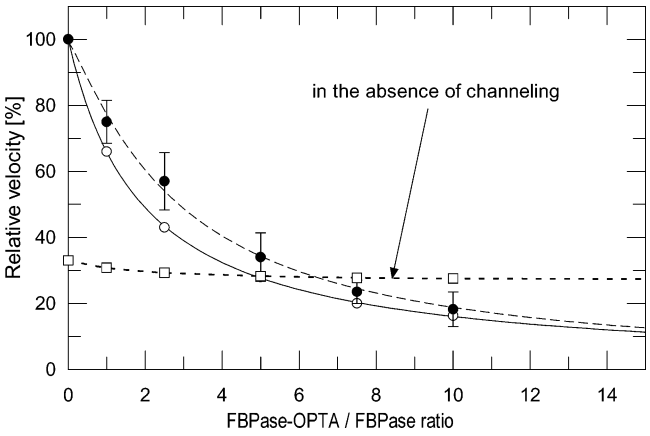


FIGURE 8: Test for channeling. Experimental data (- - -, ●) and theoretical plots of three possible effects of inactive FBPase on the rate of F6-P synthesis in the channeling test are shown. The channeling simulation (—, ○) was calculated using eq 1 and the K_a values for FBPase−aldolase and FBPase$_{OPTA}$−aldolase, presented in Table 1. In the absence of channeling, the rate of the reaction would be the same as that observed under conditions protective to the complex formation (see Figure 7a). The presence of the inactive form of FBPase should slightly affect the rate of the reaction (•••, □) as a result of the partial inhibition (∼20%) of aldolase activity upon its association with FBPase$_{OPTA}$ (see Figure 3). Every data point is the mean ± SD of three independent enzymatic activity determinations.

The reactions were carried out in the absence of AMP.

Test for Channeling. The relative activity of the aldolase−FBPase complex was significantly diminished upon the addition of FBPase$_{OPTA}$ (Figure 8), which strongly suggests the direct transfer of F1,6-P_2 from aldolase to FBPase. The experimental data-points fit well with the theoretical points calculated from eq 1 (v_C was assumed to be 100%)

$$v_D = v_C \frac{C_a K_a}{C_a K_a + C_i K_i} \quad (1)$$

where v_D is a velocity of the aldolase−FBPase complex in the presence of the inactive form of FBPase, v_C is a velocity of the aldolase−FBPase complex in the absence of the inactive form of FBPase, C_a is the concentration of unmodified FBPase, K_a is the binding constant between aldolase and unmodified FBPase, C_i is the concentration of the inactive form of FBPase, and K_i is the binding constant between aldolase and inactive FBPase.

Activity of Aldolase and FBPase in Rabbit Skeletal Muscle. The FBPase activity was approximately 7 units/g of wet rabbit muscle, which corresponds to 3.8 μM subunit concentration (assuming that specific activity is equal to 50 units/mg). The activity of aldolase was ca. 250 units/g of wet rabbit muscle.

DISCUSSION

Although the classical view of metabolism features the cell as a bag of dispersed enzymes in which intermediates diffuse freely through the cytoplasm, there is growing evidence that suggests alternatively that the intermediates in many, if not in all, pathways are transferred directly between the sequential enzymes without equilibration with the fluid of the cell (*26*−*28*, *49*).

Investigating the way of regulation of glyconeogenesis in mammalian skeletal muscles, we found that muscle aldolase interacts with muscle FBPase and desensitizes the latter enzyme toward AMP inhibition (*19*, *20*). In a recent study, using the protein exchange method, we have confirmed *in situ* that aldolase and FBPase colocalize in myocytes (*18*). The observation that aldolase interacts with FBPase presumably through residues in close vicinity to amino acids involved in the binding of substrates (Table 1) (*20*) prompted us to check if this interaction affects the kinetic properties of the complex.

Our experiments, the results of which are presented here, were designed to test the possibility of channeling of F1,6-P_2 between aldolase and FBPase. The data in Figure 8 show that inactive FBPase$_{OPTA}$ competes with native FBPase for a binding site on aldolase and decreases the activity of the aldolase−FBPase complex. The high concordance between the experimental points and the theoretically calculated values (from eq 1) clearly demonstrates that the effect of the inactive form of FBPase on the activity of the aldolase−FBPase complex may be explained only if we assume the existence of the substrate channeling between the enzymes.

The analysis of the results of the set of experiments investigating the effect of various forms of FBPase on the activity of aldolase also suggests that F1,6-P_2 is channeled from aldolase to FBPase. The release of F1,6-P_2 into the bulk phase was almost undetectable when native FBPase was incubated with aldolase under conditions facilitating the complex formation. On the other hand, native FBPase interacting with aldolase was able to hydrolyze F1,6-P_2 to F6-P in the presence of 5 μM AMP (inset of Figure 3). The same amount of free FBPase not associated with aldolase was strongly inhibited by 5 μM AMP, and the hydrolysis of F1,6-P_2 was hardly observed (Figure 3 and inset therein). Rather unexpectedly, the rate of F6-P synthesis from DHAP by the FBPase−aldolase complex was about 3 times lower than the synthesis of F1,6-P_2 (Figure 3), which suggests that the specific activity of the complex is lower than the activity of free aldolase and FBPase.

More evidence that substrate channeling occurs between aldolase and FBPase comes from experiments investigating the effect of divalent cations on the complex activity (Figure 6). An important consequence of channeling is the lack of inhibition of the aldolase−FBPase complex by high concentrations of Mg^{2+}. Although, investigating the effect of Mg^{2+} on the aldolase−FBPase coupled reaction, we found that high Mg^{2+} concentrations slightly inhibited the rate of F6-P synthesis (the complex was inhibited much more weakly than free FBPase, Figure 6), we assume that this inhibition was apparent. Divalent cations seem to play a dual role in the formation mechanism of the aldolase−FBPase complex. Within the range of 0−2 mM, magnesium ions facilitate the interaction between the enzymes (Figure 5c). It is well-documented that Mg^{2+} stabilizes the mobile loop 50−70 of FBPase in its engaged conformation, which enables the catalysis and prevents the inhibition by AMP (*50*, *51*). Because FBPase, upon its saturation with Mg^{2+}, has a higher affinity to aldolase (Figure 5c), one can hypothesize that the conformation of FBPase with the loop 50−70 in its engaged form somehow facilitates the interaction between FBPase and aldolase. In effect, aldolase together with Mg^{2+} may strongly stabilize the engaged state of the loop and desensitize muscle FBPase to AMP inhibition. When the distribution of electrostatic potential of FBPase with engaged state

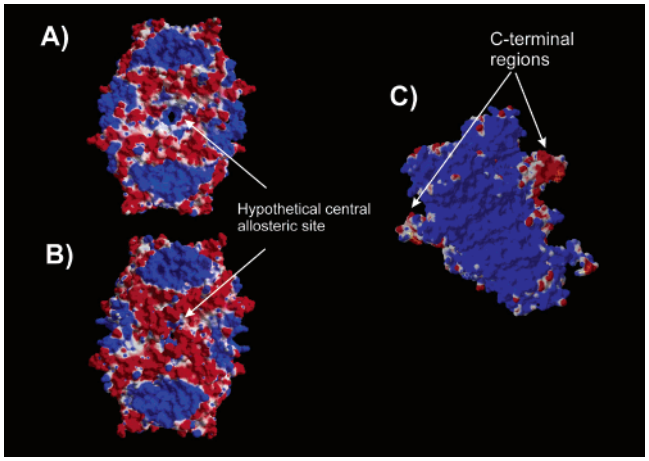


FIGURE 9: Simulation of the distribution of electrostatic potential on the molecular surface for (A) FBPase with engaged state of loop 50−70, (B) FBPase with disengaged state of the loop, and (C) for rabbit muscle aldolase (1ADO). Positively charged regions are blue, whereas the negative ones are red. The coordinates for constructing the rabbit muscle FBPase monomer with the disengaged loop were taken from the X-ray structure of porcine liver FBPase (Protein Data Bank entry 1RDY). Quaternary structure of FBPase in the T state was prepared using 1FTA. The structure of rabbit muscle FBPase with the loop in its engaged state was constructed on the basis of 1CNQ. All structures and the electron density maps were prepared using SPDB software (*59*−*61*).

of loop 50−70 is compared to that of FBPase with disengaged state of the loop (which is observed when AMP is associated with FBPase), a significant rearrangement of electron density maps is observed (Figure 9). It is presumed that the transition from the disengaged to the engaged state of the loop causes a development of a relatively homogeneous, positively charged cavity in the central region of an FBPase tetramer, which may facilitate the interaction between FBPase and the negatively charged C-terminal region of the aldolase subunit (Figure 9). Such a mode of interaction between aldolase and FBPase seems to be supported by the discovery of a new allosteric pocket located near the center of FBPase (*52*), as well as by the finding that limited proteolysis of the COOH terminus of liver aldolase abolishes its ability to form the complex with liver FBPase (*53*).

In contrast to the effect exerted by physiological concentrations of Mg^{2+} (1−2 mM), high concentrations of magnesium (over 5 mM) decreased the association of aldolase to FBPase, presumably by an increase of the ionic strength of the reaction medium (Figure 5c).

The activity of the complex in the presence of 20 mM Mg^{2+} (Figure 6) was similar to that observed when 40−60 mM concentration of monovalent cations was added to the reaction (Figure 4). The ionic strength in both cases was similar, 20−40 mM. Because the high Mg^{2+} concentration, the FBPase activator (K^+), as well as the cation, which almost does not affect FBPase activity (Na^+), influenced the rate of F6-P synthesis by the complex in a similar manner; thus, the inhibition by cations of the aldolase−FBPase activity can be assumed to result only from the decrease of the complex concentration (parts a−c of Figure 5). The destabilizing effect of the ionic strength on aldolase−FBPase association not only would support the idea that electrostatic attraction may be a driving force in the complex formation, but it would also explain the weak inhibition of the aldolase−FBPase complex activity by high concentrations of magnesium. Although the physiological concentration of KCl decreases the binding constant between aldolase and FBPase about 50 times, the interaction between the enzymes is still relatively high ($K_a = 4.2 \times 10^6$ M^{-1}), which suggests that in muscle fibers almost all (≈100%) of FBPase molecules are saturated with aldolase (eq 2). From the definition

$$K_a = [AF]/([A] - [AF]) \times ([F] - [AF]) \qquad (2)$$

$$\text{Thus, } [AF] = [(K_a[A] + K_a[F] + 1) - \sqrt{(K_a[A] + K_a[F] + 1)^2 - 4K_a^2[A][F]}]/[2K_a]$$

where $[AF]$ is the concentration of the aldolase−FBPase complex, $[F]$ is the total concentration of FBPase (∼3.8×10^{-6} M), $[A]$ is the total concentration of aldolase (∼1.3×10^{-4} M (*21*), and K_a is the association constant between aldolase and FBPase in the presence of 150 mM KCl (4.2×10^6 M^{-1}, Figure 5a).

An experiment in which the effect of various concentrations of FBPase on the activity of free aldolase, as well as of the aldolase−FBPase complex, was tested (Figure 7a) provides indirect evidence that F1,6-P_2 is channeled from aldolase to FBPase. At the lowest concentration of FBPase (34 nM) sufficient to saturate almost all aldolase in the assay, the activity of the complex was about 6 times higher than that determined for free aldolase. Additionally, the rate of F6-P synthesis by the complex was constant and independent of FBPase concentrations higher than 5 μg/mL (34 nM) (Figure 7a).

The results presented in Figure 7a might also be explained assuming the model of free diffusion of intermediates between aldolase and FBPase. However, this classical approach would require two strong assumptions to be made: (1) that aldolase activity upon its association with FBPase decreases about 4-fold, and (2) that 5 μg/mL (34 nM) of FBPase may play the role of the coupling enzyme in the assay when aldolase is saturated with FBPase. The consequence of the second assumption would be that FBPase, in the presence of aldolase, would have increased its affinity to the substrate manyfold (∼40−50 times) (Figure 7a). It would be difficult to fulfill the second assumption, which renders the classical interpretation of the results unrealistic.

The highest rate of the reaction catalyzed by free aldolase was about 4 times higher than the velocity of the complex (Figure 7a), which may suggest that, under experimental conditions, only every fourth monomer of aldolase, presumably one within the tetramer, catalyzes the reaction. The best-fitted model describing the interaction between aldolase and FBPase accounts for the existence of two conformational states of the complex (*20*). Therefore, it appears that the partial loss of activity by aldolase upon binding to active FBPase could reflect the existence of the second conformational state of the complex, in which practically only one aldolase monomer is active.

Numerous accounts indicate that, in myocytes, the enzymes of glycogen synthesis from carbohydrate precursors are localized around the Z line (*17*, *18*, *54*). On the other hand, almost all glycolytic enzymes and creatine kinase bind to actin filaments within the I band (*55*−*58*), and they probably function as a glycolytic multienzyme complex. It seems that this F-actin-based localization of glycolytic

metabolon reflects the evolutionary pressure on glycolysis to provide ATP directly to myosin ATPase [myofibrillar actin is a structural basis for the glycolytic complex in muscle tissue of phylogenetically distinct groups of animals (*55*, *56*)]. Supposedly, the synthesis of glycogen within the sarcomer, close to the glycolytic complex, also reflects the evolutionary adaptation of muscle fibers toward a more intensive contraction.

However, the localization of glyconeogenic metabolon in close proximity to the glycolytic complex raises a question concerning the compartmentation of metabolic pathways. If the channeling of the substrate between aldolase and FBPase did not occur, F1,6-P_2 molecules synthesized from carbohydrate precursors would diffuse within the sarcomeric space and most of them would be scavenged by many times more active glycolytic pathways. Thus, it seems that the substrate channeling in myofibrillar glyconeogenesis should be regarded, first of all, as an elementary mechanism enabling any activity of the pathway and, additionally, as a phenomenon that may increase the efficiency of this pathway.

Our results show that glyconeogenesis is yet another among many metabolic pathways in which substrate channeling was found (*27*−*34*). Although many interactions between enzymes are relatively weak and their complexes are not stable *in vitro*, which makes it impossible to demonstrate the channeling, the increasing number of examples of channeling may suggest that intermediate channeling is a rather common, basic mechanism of enzyme kinetics and not an evolutionary exception.

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SUPPORTING INFORMATION AVAILABLE

Effect of 10% PEG 8000 on the inhibition of rabbit muscle FBPase by AMP (Figure S1). Effect of various concentration of DHAP on the stability of aldolase−FBPase in the presence and absence of PEG 8000 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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