Muscle FBPase in a complex with muscle aldolase is insensitive to AMP inhibition

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Abstract Real-time interaction analysis, using the BIAcore biosensor, of rabbit muscle FBPase–aldolase complex revealed apparent binding constant [K_{Aapp}] values of about 4.4×10^8 M^{-1}. The stability of the complex was down-regulated by the glycolytic intermediates dihydroxyacetone phosphate and fructose 6-phosphate, and by the regulator of glycolysis and glyconeogenesis – fructose 2,6-bisphosphate. FBPase in a complex with aldolase was entirely insensitive to inhibition by physiological concentrations of AMP ($I_{0.5}$ was 1.35 mM) and the cooperativity of the inhibition was not observed. The existence of an FBPase–aldolase complex that is insensitive to AMP inhibition explains the possibility of glycogen synthesis from carbohydrate precursors in vertebrates' myocytes.

Key words: Muscle FBPase; Surface plasmon resonance; AMP inhibition; Glyconeogenesis; Dihydroxyacetone phosphate

1. Introduction

The conversion of carbohydrate precursors into glucose (gluconeogenesis) is necessary to maintain the required level of blood glucose in mammals. Gluconeogenic activity occurs mainly in liver and kidney. However, numerous studies have shown that skeletal muscles, although unable to synthesize and release glucose to blood, can synthesize glycogen from carbohydrate precursors (glyconeogenesis) [1–3].

Fructose 1,6-bisphosphatase (FBPase) [EC 3.1.3.11] is the key enzyme of gluconeogenesis and glyconeogenesis. The enzyme 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+}) [4,5]. Mammalian FBPases are activated by monovalent cations [4–6], inhibited competitively by fructose 2,6-bisphosphate (F2,6-P_2) and allosterically by AMP [4,5,7–9]. A synergistic effect of both inhibitors on FBPase has also been reported [8,10].

Liver and muscle isozymes have been found in mammalian tissues [5,11]. The basic difference between the liver and the muscle FBPase concerns their sensitivity to AMP inhibition. $I_{0.5}$ for AMP for the muscle isozyme is about 0.1 μM and this value is 50–100 times lower than the corresponding value determined for the liver isozyme [5,12–15]. Taking into account that the AMP level in muscle cell is within the range of 20–30 μM and F2,6-P_2 is ca. 1–2.5 μM, the muscle FBPase should be almost completely inhibited in vivo [10]. This raises the question of how muscle FBPase may function in vivo, and, following that, how glyconeogenesis can proceed in myocytes.

Previously we have reported that muscle aldolase decreases sensitivity of muscle FBPase to AMP inhibition; in the presence of 10 μM aldolase, $I_{0.5}$ for AMP for muscle FBPase was increased 10-fold [13].

Employing the new approach to aldolase–FBPase complex activity measurement, we demonstrate that muscle FBPase is almost completely desensitized by muscle aldolase to AMP inhibition. We also present evidence that the aldolase–FBPase complex concentration is down-regulated by low molecular weight effectors.

2. Materials and methods

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

All the chemicals used were of analytical grade.

2.1. Enzyme purification and activity determination

Rabbit muscle aldolase and FBPase were purified according to Penhoet et al. [16] and Rakus et al. [17], respectively. The activities of aldolase and FBPase were determined as described by Racker (modified) [18], and Rakus and Dzugaj [17], respectively.

Spectrophotometric measurements were performed with an HP 8452A diode array spectrophotometer. All kinetic calculations were performed using Microsoft Excel 2000 and GraFit 4 programs [19].

2.2. The activity of FBPase–aldolase complex

To determine the activity of FBPase–aldolase complex in the presence of various concentrations of AMP and F2,6-P_2 a great excess of FBPase over aldolase (1000 times) and a physiological concentration of dihydroxyacetone phosphate (DHAP) were used (80 μM DHAP was added to the reaction mixture).

To check the effect of DHAP on the rate of F6-P synthesis by aldolase–FBPase complex, various concentrations of DHAP (0–1 mM) were used.

The standard assay medium contained: 0.1 μg/ml aldolase, 100 μg/ml FBPase, 20 mM Tris, 0.25 mM EDTA, 1.25 mM $MgCl_2$, 100 mM KCl, 10% polyethylene glycol (PEG 8 000), 0.5 mM nicotinamide adenine dinucleotide phosphate, 50 U/ml of glucose 6-phosphate dehydrogenase, 50 U/ml of glucose 6-phosphate isomerase, 50 U/ml of triose 3-phosphate isomerase and 0.08 mM DHAP; pH 7.5, $T = 37$°C.

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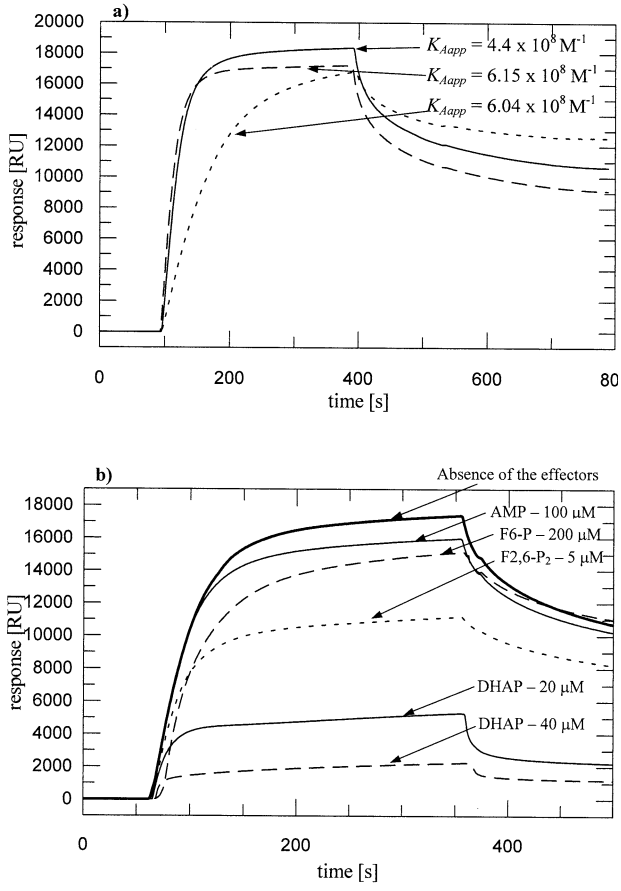

Fig. 1. a: The analysis of FBPase interactions with various concentrations of aldolase (solid line, 1.56 μM; dashed line, 1.25 μM; dotted line, 0.625 μM) by surface plasmon resonance. K_{Aapp} values were calculated using BIAevaluation 3.1 software from Biacore. Each K_{Aapp} value represents the mean of triplicate determinations. b: The influence of low molecular effectors (AMP, DHAP, F6-P, F2,6-P$_2$) on the affinity of aldolase (1.56 μM) to FBPase.

2.3. Real-time interaction analysis by BIAcore

The binding of aldolase to immobilized FBPase was measured by using BIAcore 1000 (Biacore AB, Uppsala, Sweden). The sensor chips CM5 with carboxymethylated dextran matrix were used throughout all experiments. Immobilization of rabbit muscle FBPase was carried out using the standard EDC/NHS chemical procedure for activation of a working channel. A 15 μl solution of FBPase (0.2 mg/ml in 10 mM acetate buffer, pH 6.0, flow rate 5 μl/min) was injected over the activated sensor surface, followed by a 50-μl solution of 1 M ethanolamine to deactivate the unreacted NHS-esters. The chip was equilibrated with the running buffer (5 mM HEPES, 0.05 mM EDTA, 50 mM KCl, pH 7.4, T = 25°C) until a stable baseline was obtained. All binding experiments were carried out at 25°C with a constant flow rate of 5 μl/min in the running buffer, unless otherwise specified.

To determine the affinity of aldolase to FBPase, 25 μl aliquots of various concentrations of aldolase (0.1–0.25 mg/ml in running buffer) were injected over FBPase-immobilized sensor chip (association phase), followed by a 10-min washing with the running buffer (dissociation phase). The sensor surface was then regenerated by a 3-min injection with 150 μl of 10 mM HCl to remove the aldolase.

To check the influence of low molecular effectors (AMP, DHAP, F6-P, F2,6-P$_2$) on the affinity of aldolase to FBPase, the running buffer was supplemented with appropriate concentrations of the effectors.

The analysis for the dissociation and association rate constants was performed by the BIAevaluation 3.1 software supplied by the manufacturer.

3. Results

Several binding models were used to fit the results of real-time interaction data between rabbit muscle aldolase and rabbit muscle FBPase. The best-fitted model describing the interaction accounts for the existence of one binding site on the surface of the FBPase molecule and two conformational states of the complex. A globally fitted apparent binding constant $K_{Aapp} = (k_a1/k_d1)/(k2/k3)$ (where k_a1, k_d1, $k2$ and $k3$ are, respectively, the first association and the first dissociation rate constants, and the rate constants of transformation between two conformational states of the complex) revealed that aldolase interacts with FBPase with K_{Aapp} within the range of 4.4×10^8 to 6.15×10^8 M^{-1} (Fig. 1a). Calculated, the association constant ($K_A = (k_a1/k_d1) \approx 2.6\times10^6$ M^{-1}) is much higher than that reported previously: $K_A \approx 1.15\times10^5$ M^{-1} [13]. It can be explained, at least partially, by lower salt concentration in the binding medium used in this study (−50 mM KCl), as opposed to 150 mM KCl used in our previous report.

The stability of the aldolase–FBPase complex was almost insensitive to the physiological concentration (20 μM) of AMP (data not presented) and even a 100 μM concentration of AMP decreased K_{Aapp} only about 20 times (Fig. 1b and Table 1). A stronger destabilization of the complex was observed in the presence of F2,6-P$_2$, F6-P and DHAP (Fig. 1b and Table 1). A two-fold higher than physiological, 5 μM concentration of F2,6-P$_2$ caused almost a 60-fold decrease of the affinity of aldolase to FBPase (Fig. 1b and Table 1). The influence of DHAP on the aldolase–FBPase complex was even stronger than the effect of F2,6-P$_2$, and the K_{Aapp} value

Table 1
Kinetic data on the binding of aldolase (1.56 μM) to FBPase, as measured by surface plasmon resonance – the influence of low molecular weight effectors

Effector	k_a1 [M^{-1} s^{-1}]	k_d1 [s^{-1}]	K_A [M^{-1}]	$k2$ [s^{-1}]	$k3$ [s^{-1}]	K_{Aapp} [M^{-1}]
In the absence of effectors	1.61×10^4	6.26×10^{-3}	2.57×10^6	1.96×10^{-3}	1.13×10^{-5}	4.4×10^8
	S.E.M. 25	S.E.M. 4.51×10^{-5}		S.E.M. 4.8×10^{-5}	S.E.M. 1.3×10^{-4}	
AMP-100 μM	1.98×10^4	7.61×10^{-3}	2.60×10^6	3.22×10^{-3}	4.47×10^{-4}	1.9×10^7
	S.E.M. 45	S.E.M. 4.86×10^{-5}		S.E.M. 9.57×10^{-5}	S.E.M. 1.23×10^{-4}	
F6-P-200 μM	2.01×10^4	1.46×10^{-2}	1.38×10^6	1.17×10^{-2}	3.02×10^{-3}	7.8×10^6
	S.E.M. 166	S.E.M. 3.86×10^{-4}		S.E.M. 5.65×10^{-4}	S.E.M. 4.02×10^{-5}	
F2,6-P$_2$-5 μM	2.78×10^3	1.18×10^{-2}	2.36×10^5	8.45×10^{-3}	2.63×10^{-3}	7.5×10^6
	S.E.M. 147	S.E.M. 1.59×10^{-4}		S.E.M. 2.46×10^{-4}	S.E.M. 9.59×10^{-5}	
DHAP-20 μM	3.54×10^4	4.81×10^{-2}	7.36×10^5	4.22×10^{-3}	6.98×10^{-4}	4.5×10^6
	S.E.M. 876	S.E.M. 1.27×10^{-3}		S.E.M. 1.23×10^{-4}	S.E.M. 4.69×10^{-5}	
DHAP-40 μM	1.6×10^4	4.25×10^{-2}	3.76×10^5	9.17×10^{-3}	9.9×10^{-4}	3.5×10^6
	S.E.M. 112	S.E.M. 3.72×10^{-4}		S.E.M. 7.67×10^{-5}	S.E.M. 1.12×10^{-5}	

Each K_{Aapp} value represents the mean and standard error (S.E.M.) of triplicate determinations.

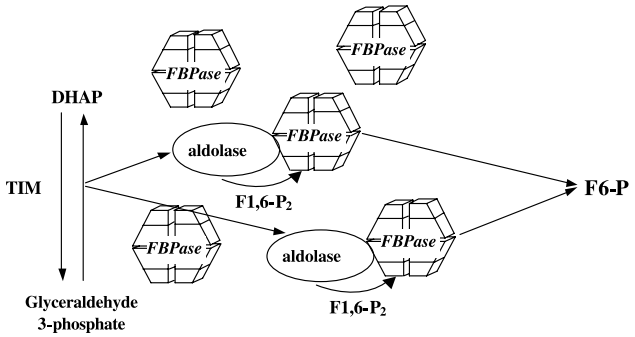

Fig. 2. In the presence of an excess of FBPase, the synthesis of F1,6-P_2 is practically catalyzed only by aldolase bound with FBPase. Synthesized F1,6-P_2 is targeted directly to FBPase, which catalyzes its hydrolysis to F6-P.

determined in the presence of 40 μM DHAP was ca. 125 times lower than K_{Aapp} determined in the absence of DHAP.

The F6-P concentration reported for a resting rabbit muscle [20] reduced the K_{Aapp} for aldolase–FBPase complex more than 50 times compared to K_{Aapp} for aldolase–FBPase in the absence of effectors (Fig. 1b and Table 1).

A standard assay for determination of FBPase activity in a complex with aldolase was described by Rakus and Dzugaj [13] as a development of the method of Morikofer-Zwez [21]. In that method a great excess of aldolase over FBPase (more than 100 times) and high concentration of DHAP (0.5 mM) were used. However, the data on the effect of DHAP on FBPase–aldolase complex stability suggest that this compound strongly destabilizes the complex. This finding explains why Rakus and Dzugaj [13] could observe only partial desensitization of FBPase by aldolase to AMP inhibition. It is presumed that under the assay conditions used in our previous report only a small part of FBPase molecules (ca. 1%) could interact with aldolase. In the present paper we used a great excess of FBPase over aldolase (1000 times) and a physiological concentration of DHAP (Fig. 2). Under such conditions more than 95% of aldolase should be saturated with FBPase during the reaction.

The response of FBPase–aldolase complex activity to the increasing concentration of DHAP was biphasic (solid line, Fig. 3a). The rate of the reaction was linear up to 200 μM concentration of added DHAP (dashed line) and the second phase of the curve describing the dependence of FBPase velocity on DHAP concentration was hyperbolic (dotted line).

FBPase in a complex with aldolase was inhibited by AMP, with $I_{0.5}$ ca. 1.35 mM and without any positive cooperativity (Fig. 3b). Contrary to the inhibition of rabbit muscle FBPase in the absence of aldolase, we could not observe any significant inhibition of the complex under AMP concentrations below 100 μM (Fig. 3b, inset).

The inhibition of FBPase–aldolase complex by F2,6-P_2 was negatively cooperative (n was 0.65), and the concentration of F2,6-P_2 which caused a two-fold inhibition of the complex was ca. 346 μM (Fig. 3c).

4. Discussion

The glycolytic and glyconeogenic enzymes have been considered as soluble constituents of the cell. However, many studies indicate that glycolytic enzymes are reversibly associated with cellular structures and this association alters their properties [22].

On the other hand, only little information is available on the regulation and spatial organization of the enzymes of glyconeogenesis. [13,23]. The basic problem of muscle glyconeogenesis is the apparent inactivity of muscle FBPase isozyme under physiological concentrations of AMP. Recently, we have shown that rabbit muscle FBPase is partially desensitized to AMP inhibition by muscle aldolase [13]. By now we have presented evidence that muscle FBPase in a complex with muscle aldolase is practically completely insensitive to AMP inhibition. In the presence of 20 mM AMP (about 1000 times higher than physiological concentration) only a five-fold inhibition of FBPase–aldolase complex was observed.

The destabilization of aldolase–FBPase complex by DHAP, F6-P_2 and F2,6-P_2, which interact with active sites of aldolase

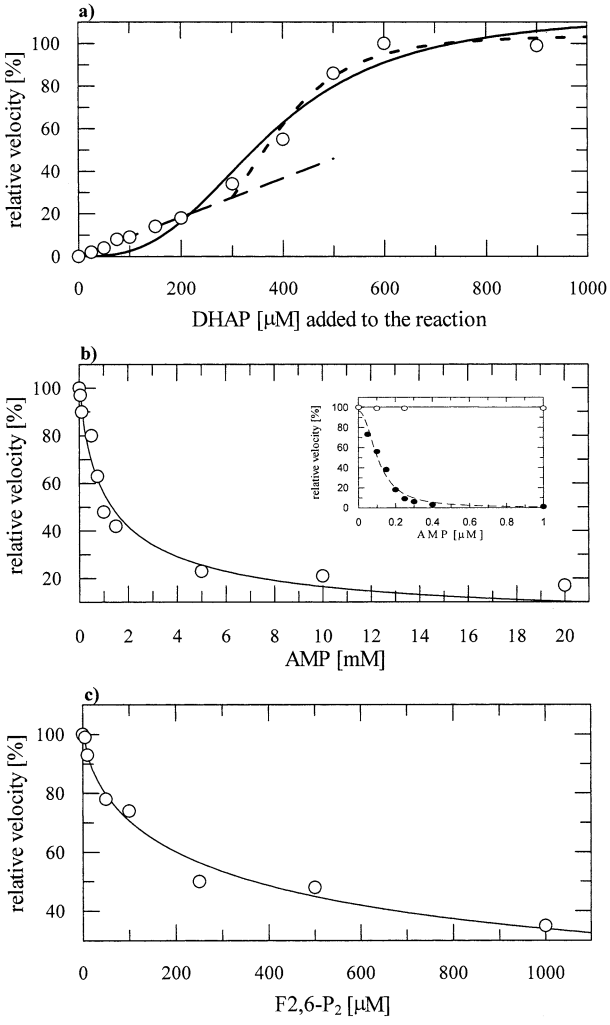

Fig. 3. a: The biphasic response of FBPase–aldolase complex activity on DHAP concentration (solid line). The rate of the reaction is linear up to 200 μM concentration of added DHAP (dashed line) and the second phase of the curve describing the dependence of FBPase velocity on DHAP concentration is hyperbolic (dotted line). b: The inhibition of aldolase–FBPase complex by AMP. In the inset, the inhibition of muscle FBPase in a complex with aldolase (solid line, empty circles) and muscle FBPase in the absence of aldolase (dashed line, full circles) are presented. c: The inhibition of FBPase–aldolase complex by F2,6-P_2.

and FBPase, was shown in the binding experiments (Fig. 1b and Table 1).

F2,6-P_2 is a competitive inhibitor of mammalian FBPases with inhibitory constant (K_i) below 1 μM [7,8,10,14,17]. In the presence of aldolase, the apparent K_i was about 350 μM. On the other hand, F2,6-P_2 significantly decreased the affinity of FBPase to aldolase: in the presence of 5 μM F2,6-P_2, the binding constant for a complex of aldolase–FBPase was lowered about 60 times. The similarity of the effects of AMP and F2,6-P_2 on the kinetics and binding properties of the complex strongly suggests a similar mechanism of the inhibition. Both AMP and F2,6-P_2 may interact with the active site of FBPase, destabilizing the aldolase–FBPase complex. Thus, AMP and F2,6-P_2 decrease the concentration of the complex and, as a result, they can inhibit FBPase activity in a classic manner.

The binding of DHAP to aldolase resulted in a dramatic decrease of aldolase affinity to FBPase – K_{Aapp} was reduced more than 100 times. The dependence of the complex activity versus increasing DHAP concentration was biphasic (Fig. 3a). Supposedly, the first phase of the curve represents the state in which almost all aldolase molecules were bound to FBPase. The activity reported during the second phase of the curve, under high concentrations of DHAP, seems to reflect the activity mainly of an unbound form of aldolase.

It is possible that within the complex the active sites of FBPase are in close vicinity to active sites of aldolase. This would enable a direct transfer, by channeling or restricted free diffusion mechanism, of F1,6-P_2 from aldolase to FBPase.

The synthesis of glycogen from lactate in myocytes takes place during recovery from exercise [2,24]. The concentrations of DHAP, F6-P and F2,6-P_2 in resting striated muscle cells are, respectively, about 25–166 μM, 100–200 μM and below 1 μM [20,25]. After stimulation of muscle contraction the concentration of these compounds immediately increases two to four times in the first 10 s of stimulation [20]. Thus, DHAP and F6-P might be the negative regulators of glyconeogenesis. F2,6-P_2 is a commonly known inhibitor of FBPase [7,8]; however, its role in the regulation of glycogen synthesis from lactate may be more complex. Supposedly, F2,6-P_2 inhibits glyconeogenesis not only by simple competitive inhibition of FBPase but also by the destabilization of the glyconeogenic complex aldolase–FBPase.

To conclude, the results presented in our report demonstrate that muscle FBPase bound with aldolase is entirely desensitized to AMP inhibition, which enables glycogen synthesis from carbohydrate precursors in myocytes. The stability of aldolase–FBPase complex is down-regulated by DHAP, F6-P and F2,6-P_2 – the compounds whose concentration increases significantly and immediately during physical exercise. Since DHAP, F2,6-P_2 and F6-P bind to the active sites of the enzymes, channeling or a restricted free diffusion mechanism of F1,6-P_2 transfer from aldolase to FBPase may be expected.

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