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Research Communication

Muscle-Type 6-Phosphofructo-1-kinase and Aldolase Associate Conferring Catalytic Advantages for Both Enzymes

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Summary

6-Phosphofructo-1-kinase (PFK) and aldolase are two sequential glycolytic enzymes that associate forming heterotetramers containing a dimer of each enzyme. Although free PFK dimers present a negligible activity, once associated to aldolase these dimers are as active as the fully active tetrameric conformation of the enzyme. Here we show that aldolase-associated PFK dimers are not inhibited by clotrimazole, an antifulgal azole derivative proposed as an antineoplastic drug due to its inhibitory effects on PFK. In the presence of aldolase, PFK is not modulated by its allosteric activators, ADP and fructose-2,6-bisphosphate, but is still inhibited by citrate and lactate. The association between the two enzymes also results on the twofold stimulation of aldolase maximal velocity and affinity for its substrate. These results suggest that the association between PFK and aldolase confers catalytic advantage for both enzymes and may contribute to the channeling of the glycolytic metabolism. © 2011 IUBMB

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Keywords glycolysis; phosphofructokinase; aldolase; clotrimazole; channeling.

INTRODUCTION

6-Phosphofructo-1-kinase (PFK; phosphofructokinase; EC 2.7.1.11) is the major regulatory glycolytic enzyme and acts as the pacemaker of glycolysis (*1*). This highly regulated enzyme exists in diverse oligomeric conformations, including monomers, dimers, tetramers, and hexadecamers (*1*). The transition between dimers and tetramers is highly relevant for the enzyme's regulation because the former have very low catalytic activity, whereas the latter have been described as fully active (*1–3*). Several allosteric modulators of PFK affect the equilibrium between dimers and tetramers; the inhibitors citrate and lactate favor the formation of dimers, and the activators ADP and fructose-2,6-bisphosphate (F2,6BP) stabilize tetramers (*4–8*). Moreover, the association of PFK with aldolase or calmodulin stabilizes PFK in a dimeric conformation that has a catalytic activity equivalent to that of the tetramers (*4, 9–11*).

Aldolase (EC 4.1.2.13), the sequential enzyme to PFK on glycolysis, cleaves the product of the PFK reaction, fructose-1,6-bisphosphate (F1,6BP), into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The association between PFK and aldolase contributes to the channeling of glycolysis, increasing the rate of this pathway (*12–15*). Aldolase and PFK associate with the cytoskeleton (*9, 16–23*), especially in tumor cells (*19, 20*), which increases their activity and the channeling of glycolysis (*14*). This event is directly correlated to the Warburg effect, conferring invasive metastatic properties to the tumor (*24, 25*). The down-regulation of both PFK and aldolase has been reported to drastically decrease tumor cell viability (*20*).

Clotrimazole (CTZ) is an antifungal derivative azole with calmodulin antagonist properties and described as a potential antineoplasic drug due to its ability to decrease tumor cell glycolysis (*20, 26–28*). We have previously reported that this drug decreases the association of PFK and aldolase with the tumor cell cytoskeleton, thus decreasing tumor viability (*20*). Moreover, CTZ directly inhibits PFK by promoting the dissociation of PFK tetramers into dimers (*23*) and by augmenting the inhibitory effectiveness of ATP (*29*).

The present work aimed to evaluate whether the interaction of PFK and aldolase interferes with their catalytic activities and the inhibition of the enzymes by CTZ.

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MATERIALS AND METHODS

Materials

ATP, ADP, CTZ, citrate, F1,6BP, F2,6BP, F6P, lactate, NADH, triosephosphate isomerase, and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical (St. Louis, MO). ^{32}Pi was purchased from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ-^{32}P]ATP was prepared according to Maia et al. (*30*). PFK was purified from rabbit skeletal muscle, as previously described (*21*). Aldolase from rabbit skeletal muscle was purchased from Sigma Chemical (St. Louis, MO). All protein content measurements were performed as described by Lowry et al. (*31*).

PFK Activity

PFK activity was measured by the method described by Sola-Penna et al. (*32*) with the modifications introduced by Zancan and Sola-Penna (*33, 34*). The reaction medium contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (4 μCi/nmol), 1 mM fructose-6-phosphate and 1 μg/mL PFK, except when otherwise specified. The reaction was stopped after increasing reaction times by the addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol, and after centrifugation, the supernatant containing [1-^{32}P]fructose-1,6-bisphosphate was analyzed in a liquid scintillation counter. The signals from appropriate blanks in the absence of fructose-6-phosphate were measured and subtracted from all measurements to account for ATP hydrolysis. The catalytic rate was calculated by linear regression of the amount of F1,6BP formed versus reaction time. The enzyme activity was expressed as mU of PFK activity, where 1 mU was taken to be the formation of 1 nmol of fructose-1,6-bisphosphate per minute of reaction.

Aldolase Activity

Aldolase activity was assessed through a coupled enzyme linked assay in a reaction medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM F1,6BP, 2 mM NADH, triosephosphate isomerase, and α-glycerophosphate dehydrogenase. The reaction was followed spectrophotometrically at 340 nm, which corresponds to the absorbance of NADH. Aldolase activity was expressed as mU, where 1 mU was considered to be the reduction of 2 nmol of NADH per minute of reaction.

Intrinsic Fluorescence Measurements

Intrinsic fluorescence measurements of PFK were performed as described previously (*29*) using the same conditions described for the radioassay. Excitation wavelength was fixed at 280 nm, and fluorescence emission was scanned from 300 to 400 nm. The center of mass of the intrinsic fluorescence spectra (CM) was calculated using:

$$\mathrm{CM} = \frac{\sum \lambda \times I_\lambda}{\sum I_\lambda} \tag{1}$$

where λ is the wavelength and I_λ is the fluorescence intensity at a given λ. Center of mass is used to evaluate the oligomeric state of PFK because the dissociated enzyme exposes its tryptophans to the aqueous milieu to a greater extent than the oligomer; thus, the fluorescence emitted by these tryptophans is of lower energy. Consequently, the center of mass of a population of tetramers is smaller than that of a population of dimers, as confirmed in many recent publications (*4–6, 11, 23, 29, 35*).

Light Scattering Measurements

Light scattering measurements were performed as described previously (*6*) using the same conditions described for the radioassay. Appropriate reference spectra were subtracted from the data to correct for background interferences. The excitation wavelength was set at 510 nm and the emission light scattered was scanned from 500 to 520 nm. The total concentration of protein in the assay was 1 μg/mL.

Statistics and Calculations

Statistical analyses and enzyme kinetics calculations were performed using the software SigmaPlot 10.0 integrated with SigmaStat 3.51 (Systat, CA). Student's *t*-tests or one-tailed ANOVAs were used to evaluate the significance of the results. $P < 0.05$ was considered to be statistically significant.

The kinetic parameters for the inhibitory effects of CTZ on PFK and aldolase were calculated as described previously (*29*) by nonlinear regression using the equation

$$v = \frac{v_0 \cdot I_{0.5}^n}{I_{0.5}^n + [\mathrm{CTZ}]} + v_\mathrm{R} \tag{2}$$

where v is the calculated enzyme's activity at a given concentration of CTZ ([CTZ]), v_0 is the difference between the enzyme's activity in the absence of CTZ and the activity when CTZ is promoting its maximal inhibitory effects (v_R), $I_{0.5}$ is the inhibition constant equivalent to the concentration of CTZ resulting in 50% of the maximal inhibition (v_R) and n is the cooperativity index.

Kinetic parameters for the effects of ATP on PFK were calculated considering the two components for PFK modulation by this metabolite. The first component is the stimulatory component for the substrate saturation curve, in which PFK exhibits an allosteric pattern that is described by the equation:

$$v = \frac{\mathrm{V}_{\mathrm{max}_{\mathrm{app}}} \times [\mathrm{ATP}]^{n_\mathrm{s}}}{K_{0.5}^{n_\mathrm{s}} + [\mathrm{ATP}]^{n_\mathrm{s}}} \tag{3}$$

where v is the PFK activity at a given concentration of ATP ([ATP]), $V_{\mathrm{max}_{\mathrm{app}}}$ is the apparent maximal velocity calculated, $K_{0.5}$ is the affinity constant for this component and n_s is the cooperativity index for this component. The second component is the inhibitory component that can be adjusted by the equation:

$$v = \frac{V_\mathrm{sat} \times I_{0.5}^{n_i}}{I_{0.5}^{n_i} + [\mathrm{ATP}]^{n_i}} \tag{4}$$

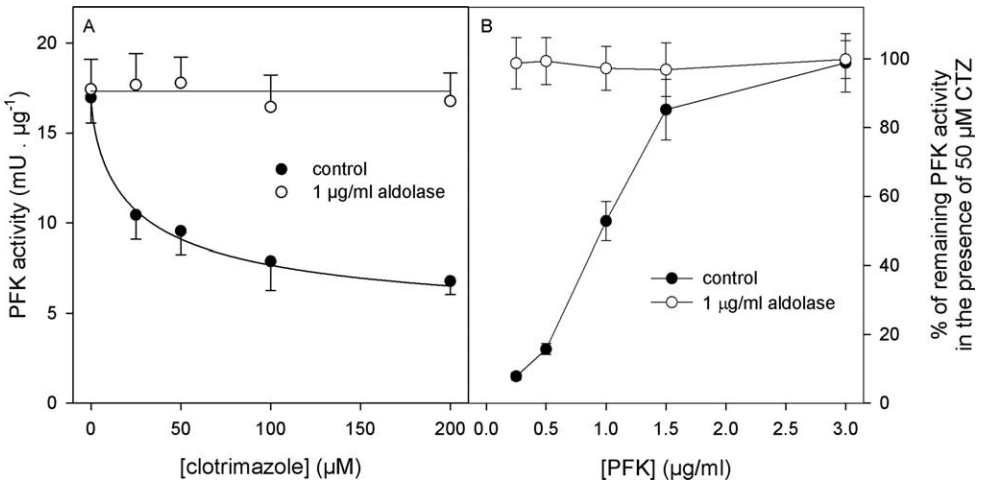

Figure 1. Effects of CTZ on PFK activity in the absence and presence of aldolase. PFK activity was assessed as described under "Materials and Methods" in the absence and in the presence of 1 μg/mL aldolase. Panel A: Dose-response curve of CTZ effects on PFK activity in the absence (control, filled circles) and in the presence of 1 μg/mL aldolase (empty circles). Solid line shown for the absence of aldolase was obtained fitting Eq. (2) to the experimental data. Solid line for the presence of aldolase is the linear regression of the plotted data. Panel B: Relative remaining PFK activity in the presence of 50 μM CTZ assessed with the concentrations of PFK indicated on the abscissa. The experiments were performed in the absence (control, filled circles) and in the presence of 1 μg/mL aldolase (empty circles). All plotted data are mean $\pm$ standard errors of, at least, four independent experiments ($n = 4$).

where v is the PFK activity at a given concentration of ATP ([ATP]), $I_{0.5}$ is the affinity constant for this component, n_i is the cooperativity index for this component and V_{sat} is the PFK activity when the first component is saturated. Assuming this statement, V_{sat} is a function of the first component of the curve and can be substituted by Eq. (3) to result in the following equation:

$$v = \frac{\frac{V_{max_{app}} \times [ATP]^{n_s}}{K_{0.5}^{n_s} + [ATP]^{n_s}} \times I_{0.5}^{n_i}}{I_{0.5}^{n_i} + [ATP]^{n_i}} \quad (5)$$

which was fitted to the experimental data through nonlinear regression for the effects of ATP on PFK activity.

Kinetic parameters for the effects of F6P on PFK were calculated through nonlinear regression using the experimental data to fit the parameters of the equation:

$$v = \frac{V\text{max} \times [F6P]^n}{K_{0.5} + [F6P]^n} \quad (6)$$

where v is the PFK activity calculated for a given concentration of F6P ([F6P]), V_{max} is the maximal velocity calculated at saturating concentrations of F6P, $K_{0.5}$ is the affinity constant for F6P, which is equal to the concentration of F6P responsible for half-activation of the PFK by F6P, and n is the cooperativity index for this phenomenon.

Kinetic parameters for aldolase were assessed using Eq. (6), substituting F6P by F1,6BP.

The kinetic parameters for the stimulatory effects of PFK on aldolase activity were calculated by nonlinear regression using the equation

$$v = v_i + \frac{v_a \cdot [PFK]^n}{Ka^n + [PFK]^n} \quad (7)$$

where v is the calculated aldolase activity at a given concentration of PFK ([PFK]), v_i is the aldolase activity in the absence of PFK, Ka is the activation constant reflecting the concentration of PFK that results in 50% of the maximal activation (v_a) and n is the cooperativity index.

RESULTS

Counteraction of CTZ-Induced Inhibition of PFK by Aldolase

PFK is inhibited by CTZ in a dose-dependent manner, with an $I_{0.5}$ of 28 $\pm$ 2 μM and a maximal inhibition of 70% (Fig. 1A, filled circles). However, when these experiments were performed in the presence of 1 μg/mL aldolase, no inhibition of PFK activity was observed up to 200 μM CTZ (Fig. 1A, empty circles). We have reported that PFK inhibition by CTZ is due to the ability of the drug to promote the dissociation of the fully active tetrameric conformation of the enzyme into relatively inactive dimers (*23, 29*). It has been reported that aldolase binds to PFK, stabilizing the enzyme in the dimeric conformation.

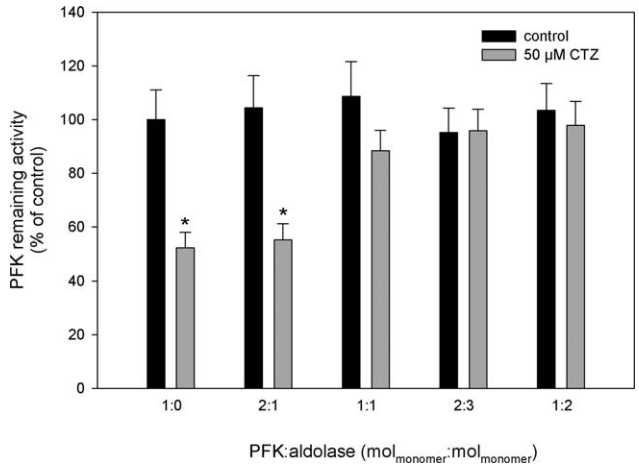

Figure 2. Reversal of CTZ-induced inhibition of PFK by aldolase. PFK activity was assessed as described under "Materials and Methods" in the presence of 1 μg/mL PFK and increasing concentrations of aldolase. The molar ratio was calculated considering PFK monomer presenting 85 kDa and aldolase monomer 45 kDa. Experiments were performed in the absence and in the presence of 50 μM CTZ as indicated. Bars are mean $\pm$ standard errors of, at least, four independent experiments ($n = 4$). *$P < 0.05$ comparing with control experiments in the absence of CTZ (Student's *t*-test).

However, these PFK dimers bound to aldolase maintain a catalytic activity similar to that observed for the tetramers (*10*). This fact could explain the lack of PFK inhibition by CTZ in the presence of aldolase, as PFK dimers formed due to the effects of CTZ would bind to aldolase and thus maintain their catalytic activity. To corroborate this hypothesis, we evaluated the effects of CTZ on PFK activity at different concentrations of the enzyme.

It has been reported that the equilibrium between PFK dimers and tetramers is directly affected by the concentration of the enzyme; the more concentrated the enzyme is, the more stable tetramers are (*6, 11*). In fact, when increasing the concentration of PFK in the reaction medium, a progressive decrease of the inhibition promoted by CTZ is observed. For example, in the presence of 1.5 μg/mL PFK, no significant effect of CTZ was observed (Fig. 1B, filled circles). Moreover, aldolase was able to abrogate the effects of CTZ on PFK at all PFK concentrations used (Fig. 1B, empty circles). Furthermore, we determined that 1 mol of aldolase monomer per mol of PFK monomer is required for aldolase to protect PFK from the inhibitory effects of CTZ. This can be observed in Fig. 2, which shows that a stoichiometry of 2 PFK monomers per aldolase monomer does not alter the inhibition of PFK by CTZ. However, when aldolase is present at a concentration proportionally equal to or in excess of that of PFK, full protection from CTZ is observed (Fig. 2). These data support the hypothesis that aldolase binds to the PFK dimers formed due to the effects of CTZ, turning these dimers into an active dimeric conformation and thus preventing the inhibitory effects of the drug.

Structural Evidences for the Counteraction of CTZ-Induced PFK Inhibition by Aldolase

Evidences for the interaction between PFK and aldolase were assessed through two distinct techniques: intrinsic fluorescence emission spectroscopy and light scattering. We have efficiently applied these methods to evaluate the transition between PFK dimers and tetramers (*4–6, 8, 11, 18, 22, 23, 29, 35, 36*). Intrinsic fluorescence measurements show a slight red-shift of PFK intrinsic fluorescence emission spectrum in the presence of aldolase (Fig. 3A, cf. the black line for PFK with the green line for PFK in the presence of aldolase). This effect becomes clear calculating the center of mass of these spectra. The center of mass of the intrinsic fluorescence emission spectra of PFK alone is 338.2 $\pm$ 0.4 nm (mean $\pm$ standard error of four independent experiments; $n = 4$). In the presence of aldolase, the calculated center of mass of PFK intrinsic fluorescence emission spectra shifts to 341.6 $\pm$ 0.5 nm ($P < 0.05$ comparing to control in the absence of aldolase; Student's *t*-test; $n = 4$). This later value is not different from the center of mass of PFK intrinsic fluorescence spectra in the presence of CTZ or aldolase and CTZ (341.3 $\pm$ 0.4 nm and 341.4 $\pm$ 0.4 nm, respectively). This red-shift observed is characteristic of the dissociation of the tetramers of the kinase into dimers, as well documented in previous publications (*4–6, 8, 11, 18, 22, 23, 29, 35, 36*). It cannot be due to the simple interference of aldolase intrinsic fluorescence emission since the signal for this emission is negligible when compared to PFK signal (Fig. 3A, cf. the red line for aldolase with the black line for PFK). Moreover, the presence of 50 μM CTZ, which has been demonstrated to dissociate PFK tetramers into dimers (*23, 29*), induced a similar effect on PFK intrinsic fluorescence emission spectrum (Fig. 3A, yellow line). This pattern is not modified in the simultaneous presence of aldolase and CTZ (Fig. 3A, blue line). These results suggest that both aldolase and CTZ stabilize the dimeric conformation of PFK.

To corroborate this hypothesis, we evaluated the ability of the proteins to scatter light, which is proportional to the dimension of the protein particles in solution. As expected, PFK scatters more light than aldolase (Fig. 3B, cf. the black line, for PFK, with the red line, for aldolase), which is compatible with the higher molecular weight of the former comparing to the later (340 kDa for PFK tetramers vs. $\sim$ 180 kDa for aldolase tetramers). CTZ strongly promotes the attenuation of the light scattered by PFK (Fig. 3B, yellow line), which is indicative of the dissociation of the enzyme. On the other hand, CTZ almost did not affected light scattering by aldolase (Fig. 3B, magenta). The simultaneous presence of PFK and aldolase in the medium promotes a pattern of light scattering (Fig. 3B, green line) that is intermediate between those observed with PFK alone and in the presence of CTZ. This is indicative that the protein particles under this conditions present an intermediate size between the

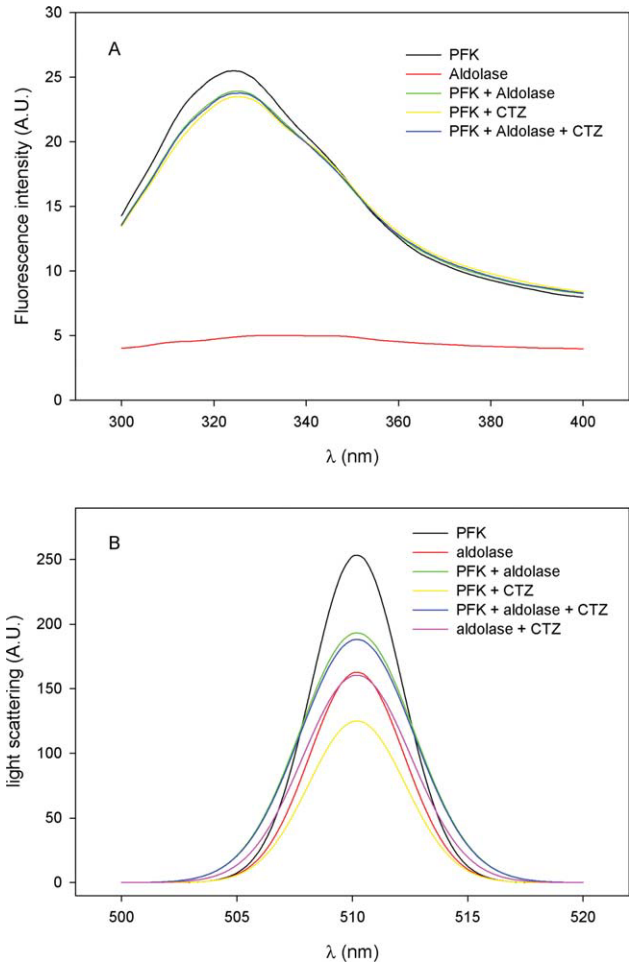

Figure 3. Structural evaluation of PFK and aldolase in the absence and the presence of CTZ. Panel A: intrinsic fluorescence emission of the enzymes was evaluated as described under "Materials and Methods" exciting the samples at 280 nm and the fluorescence emission was scanned from 300 to 400 nm and represented as arbitrary units (A.U.). The curves are representative spectra of four independent experiments. Panel B: light scattering experiments were performed as described under Materials and Methods, exciting the samples at 510 nm and recording the light scattered from 500 to 510 nm, which is represented as arbitrary units (A.U.). The curves are representative light results of four independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tetrameric and dimeric PFK conformations. Moreover, the simultaneous presence of PFK and aldolase produces protein particles that scatter more light than aldolase alone and less than PFK alone. Together with the results described above, this result suggests that PFK and aldolase might associate into heterotetramers formed by a PFK and an aldolase dimer. The ability of PFK and aldolase to generate such an heterotetramer have been demonstrated before, using other techniques (*10*). This heterotetramer, presenting ~260 kDa, would be compatible with the light scattering pattern observed in the simultaneous presence of PFK and aldolase (Fig. 3B, green line). This heterotetramer does not dissociate in the presence of CTZ, since the drug did not affect the light scattering pattern of the associated proteins (Fig. 3B, blue line). These results suggest that CTZ is not able to inhibit PFK in the presence of aldolase because it is unable to dissociate the active heterotetrameric enzyme complex formed by the two enzymes.

CTZ Affects the Kinetic Parameters of PFK, but not in the Presence of Aldolase

The kinetic parameters for PFK activation by its substrates were evaluated in the presence of CTZ and aldolase. As previously reported (*29*), CTZ alters the effects of the substrates on enzyme kinetics (Figs. 4A and 4C), decreasing the maximal velocity and the affinity of PFK for F6P and ATP at the catalytic site, while increasing the affinity of the enzyme for ATP at its inhibitory site (Table 1). In the presence of aldolase, the affinity of PFK for its substrate at the catalytic site is also lower when compared to the control in the absence of aldolase (Table 1). This occurs in spite of the fact that the maximal velocity of PFK and the affinity of ATP inhibitory site are not altered by aldolase (Table 1). On the other hand, in the presence of aldolase, CTZ presented no effect on PFK activation by its substrates (Figs. 4B and 4D; Table 1). The diminished affinity for ATP and F6P at PFK catalytic site is characteristic of the dimeric conformation of the enzyme (1) and is observed in the presence of both, CTZ or aldolase.

Allosteric Modulators of PFK Interfere with the Counteraction of PFK Inhibition by Aldolase

We evaluated the effects of CTZ on PFK activity in the presence of other modulators of the enzyme's quaternary structure, in both the absence and the presence of aldolase. For this, we used ADP and fructose-2,6-bisphosphate (F2,6BP), two stabilizers of the tetrameric structure of the enzyme, and citrate and lactate, two stabilizers of the enzyme dimers (*4–6, 8*). In the absence of aldolase (Fig. 5A), both stabilizers of PFK tetramers attenuated the inhibitory effects of CTZ. ADP was much more efficient than F2,6BP in both activating PFK and protecting the enzyme against the inhibitory effects of 50 μM CTZ; ADP promotes a fourfold stimulation of the enzyme activity and abrogates the effects of CTZ. F2,6BP increased the enzyme activity by 30% and attenuated the enzyme inhibition to 25%, versus 50% for the control (Fig. 5A). The stimulatory effects of F2,6BP are more pronounced when the enzyme is inhibited by ATP (*5, 6*), which is not the case since the ATP concentration in this experiment is not inhibitory. Therefore, since these experiments were performed in the presence of 1 mM ATP, which is not inhibitory for the enzyme (*5, 6*), it is expected that ADP would be more effective than F2,6BP in activating the enzyme and, thus, in counteracting the inhibitory effects of CTZ. When these experiments were performed in the presence of citrate, which

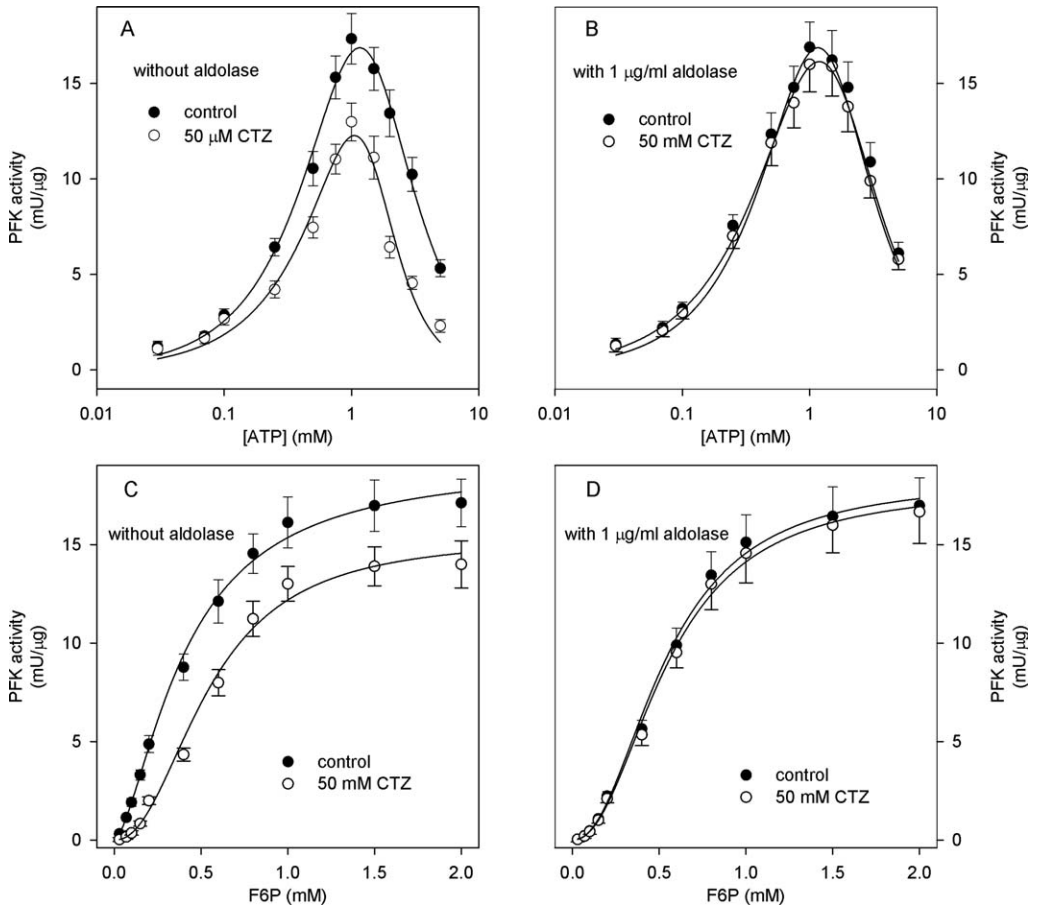

Figure 4. Effects of aldolase and CTZ on the modulation of PFK by its substrates. PFK activity was assessed as described under Materials and Methods in the absence and in the presence of 50 μM CTZ, as indicated. Panels A and D are, respectively, the ATP and F6P curves assayed in the absence of aldolase. Panels B and D are, respectively, the ATP and F6P curves assayed in the presence of 1 μg/mL aldolase. Plotted values as mean $\pm$ standard error of four independent experiments. Solid lines are the results of the adjust of Eq. (5) (panels A and C) and Eq. (6) (panels B and D) to the experimental plotted data.

Table 1
Kinetic parameters for PFK modulation by its substrates

		Control	50 μM CTZ	1 μg/mL aldolase	1 μg/mL aldolase + 50 μM CTZ
ATP	$K_{0.5}$ (mM)	0.31 $\pm$ 0.03	0.79 $\pm$ 0.06[a]	0.75 $\pm$ 0.05[a]	0.75 $\pm$ 0.07
	n_s	1.1 $\pm$ 0.2	2.0 $\pm$ 0.3[a]	1.8 $\pm$ 0.2[a]	1.9 $\pm$ 0.3
	$V_{max_{app}}$ (mU/μg)	15.2 $\pm$ 1.7	11.6 $\pm$ 1.3[a]	15.8 $\pm$ 1.6	15.8 $\pm$ 1.5
	$I_{0.5}$ (mM)	3.2 $\pm$ 0.3	1.5 $\pm$ 0.2[a]	2.9 $\pm$ 0.3	3.0 $\pm$ 0.3
	n_i	1.7 $\pm$ 0.2	1.2 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.2
F6P	V_{max} (mU/μg)	16.6 $\pm$ 1.4	12.2 $\pm$ 1.3[a]	16.5 $\pm$ 1.6	16.4 $\pm$ 1.6
	$K_{0.5}$ (mM)	0.30 $\pm$ 0.03	0.49 $\pm$ 0.03[a]	0.50 $\pm$ 0.04[a]	0.51 $\pm$ 0.05
	n	1.1 $\pm$ 0.1	2.2 $\pm$ 0.2[a]	1.7 $\pm$ 0.3[a]	1.7 $\pm$ 0.2

The parameters were calculated as described under "Materials and Methods" section, using Eq. (5) to calculate the parameter for ATP curve and equation (6) for F6P curve.

[a] $P < 0.05$ compared to control (Student's t-test).

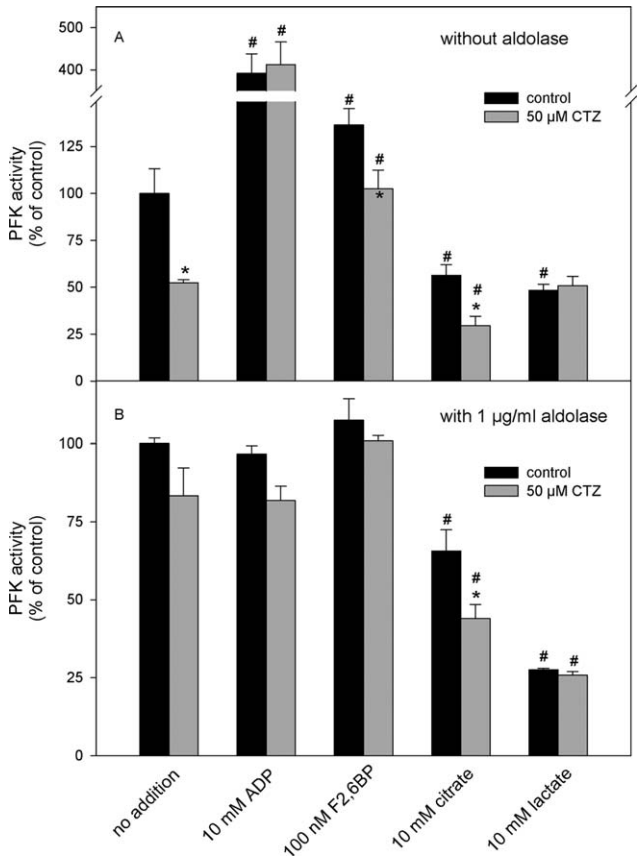

Figure 5. Influence of allosteric modulators of PFK on CTZ-induced inhibition of PFK in the absence and presence of aldolase. PFK activity was assessed as described under "Materials and Methods" in the absence and in the presence of 50 μM CTZ, as indicated. The allosteric modulators of PFK were added at the concentrations indicated on the abscissa, in the absence (Panel A) and in the presence of 1 μg/mL aldolase (Panel B). Bars are mean $\pm$ standard errors of, at least, four independent experiments ($n = 4$). *$P < 0.05$ comparing with control experiments in the absence of CTZ (Student's *t*-test). #$P < 0.05$ comparing with the respective bar where no allosteric modulator was added ("no addition"; Student's *t*-test).

inhibits the enzyme, favoring the formation of dimers (*4*), the effects of CTZ were enhanced, suggesting an additive effect (Fig. 5A). On the other hand, lactate was not able to reinforce the inhibitory effects of CTZ, despite the fact that both CTZ and lactate inhibit the enzyme by stabilizing the dimeric conformation (*4, 8, 23, 29*). Curiously, 10 mM lactate alone resulted in 52% $\pm$ 3% inhibition of PFK activity, which was equivalent to the effects of 50 μM CTZ (48% $\pm$ 2%) or lactate and CTZ together (51% $\pm$ 5%). Two hypotheses may explain this phenomenon: a) lactate and CTZ bind to the same site at the enzyme; or b) the binding of one blocks the effect of the other.

In the presence of 1 μg/mL aldolase (a molar ratio of 1:2 PFK:aldolase), the above pattern changes. ADP and F2,6BP did not activate PFK (Fig. 5B), and CTZ did not cause the typical inhibitory effects in the presence of the two PFK activators (Fig. 5B). These results can be explained by the formation of active PFK dimers in the presence of aldolase. Since ADP and F2,6BP activate PFK, favoring the formation of tetramers, their effects were not seen when active PFK dimers were stabilized by aldolase. In a reciprocal way, since these dimers were active, CTZ was not able to inhibit the enzyme because the drug promotes the dissociation of the active tetramers into inactive dimers. In conclusion, ADP, F2,6BP and CTZ did not interfere with the formation of active PFK dimers induced by aldolase. On the other hand, the presence of aldolase did not prevent PFK inhibition by citrate or lactate (Fig. 5B). The inhibitory effects of citrate occur to the same extent in the absence or in the presence of aldolase. However, lactate is a more effective inhibitor of PFK in the presence of aldolase than in its absence (Fig. 5). Moreover, in the presence of aldolase, citrate enhanced the inhibitory effects of CTZ on PFK, while in the presence of lactate, there was no additional effect of CTZ on PFK inhibition (Fig. 5B), mirroring the results observed in the absence of aldolase (Fig. 5A). In the presence of aldolase, the inhibitory effects of 10 mM lactate were more pronounced than in the absence of the enzyme (50% vs. 75% inhibition in the absence vs. presence of aldolase, respectively). The fact that the presence of aldolase did not interfere with the inhibition promoted by citrate but accentuated the inhibitory action of lactate suggests that these inhibitors have different mechanisms. It is well demonstrated that both lactate and citrate stabilize the dimeric conformation of PFK (*4, 8*), as does aldolase (*10*). Therefore, a possible explanation for the fact that aldolase potentiates the inhibitory effects of lactate but not of citrate is that lactate binds preferentially to PFK dimers. Because PFK dimers are already formed in the presence of aldolase, lactate would bind more easily and thus be more effective. Moreover, these results answer the question raised above concerning the lack of additive effects for lactate and CTZ; we can infer that the binding of lactate to PFK blocks the effects of CTZ.

PFK Stimulates Aldolase Activity

CTZ has also been shown to affect the activities of other glycolytic enzymes, such as hexokinase (*26, 35*) and aldolase (*20*). Therefore, we decided to evaluate the effects of CTZ on aldolase activity in the absence and the presence of PFK. CTZ results in the dose-dependent inhibition of aldolase in the absence and in the presence of PFK (Fig. 6A). Surprisingly, the presence of 1 μg/mL PFK in the reaction medium resulted in a twofold stimulation of aldolase activity, which persisted in the presence of all concentrations of CTZ tested (Fig. 6A). To our knowledge, this is the first time that stimulation of aldolase activity by PFK has been reported. Despite stimulating aldolase activity, PFK is not able to prevent CTZ-induced inhibition of aldolase. The effects of CTZ on aldolase reached a maximal inhibition of 98% $\pm$ 2% and followed a parallel pattern under

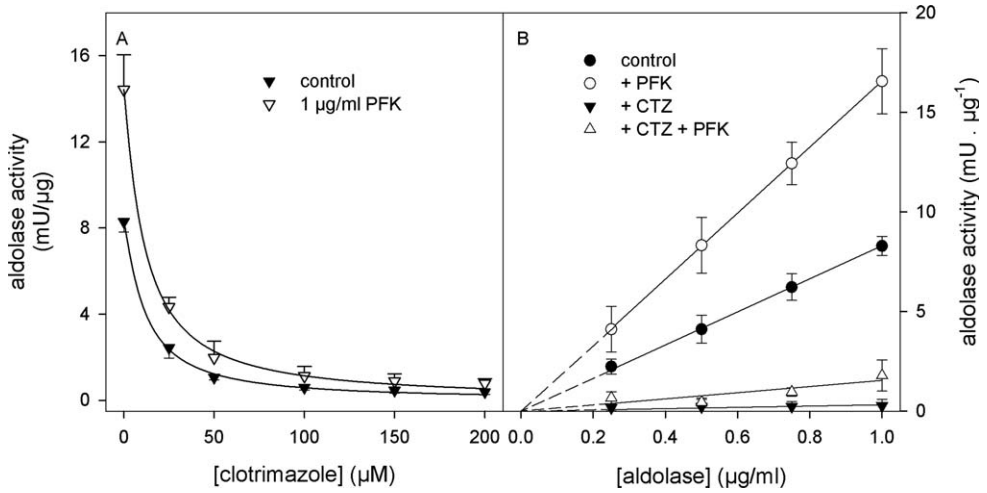

Figure 6. Effects of CTZ on aldolase activity in the absence and presence of PFK. Aldolase activity was assessed in the absence and in the presence of 1 μg/mL PFK, as described in the "Materials and Methods" section. Panel A: Dose-response curve of CTZ effects on aldolase activity in the absence (control, filled triangles) and in the presence of 1 μg/mL PFK (empty triangles). Solid lines were obtained by fitting Eq. (2) to the experimental data. Panel B: aldolase activity assessed using the concentrations of aldolase indicated on the abscissa. The experiments were performed with no further additions (control, filled circles), in the presence of 1 μg/mL PFK (empty circles), in the presence of 50 μM CTZ (filled triangles) and in the presence of 1 μg/mL PFK and 50 μM CTZ (empty triangles). Solid lines are the result of the linear regression of the plotted data, and dashed lines are the extrapolation of the linear regression results. All plotted data are mean $\pm$ standard errors of at least four independent experiments ($n = 4$).

both conditions, presenting $I_{0.5}$ values of 11 $\pm$ 1 μM and 10 $\pm$ 1 μM in the absence and presence of PFK, respectively. These values are not statistically different, suggesting that the presence of PFK does not affect the inhibition of aldolase by CTZ. The inhibitory effects of CTZ on aldolase activity were not influenced by the concentration of the enzyme in the reaction medium (Fig. 6B, cf. filled circles with filled triangles for control and for the presence of 50 μM CTZ, respectively). The presence of the drug resulted in $\sim$95% inhibition of aldolase activity in the absence and in the presence of PFK for all concentrations of aldolase tested (Fig. 6B). However, comparing the effects of CTZ on aldolase activity in the absence and the presence of PFK, it is clear that in the latter condition aldolase activity was doubled when compared to the former (Fig. 6B, cf. filled triangles and empty triangles for the absence and the presence of PFK, respectively).

To evaluate the activator property of PFK on aldolase, we assessed the aldolase activity in the presence of increasing concentrations of PFK. Aldolase activity increased with increasing concentrations of PFK in a dose-dependent manner (Fig. 7), reaching a maximal activation of 95% $\pm$ 8% and an activation constant (Ka) of 0.45 $\pm$ 0.03 μg/mL ($\sim$5.3 nM for PFK monomers). These parameters, obtained in the presence of 50 μM CTZ, are not statistically different from the control (93% $\pm$ 9% activation and Ka = 0.48 $\pm$ 0.05 μg/mL; $P > 0.05$, Student's t-test). The maximal activation of aldolase by PFK was reached in the presence of 1 μg/mL PFK (Fig. 7), representing a molar ratio of 2:1 (aldolase:PFK, based on the monomers of both enzymes).

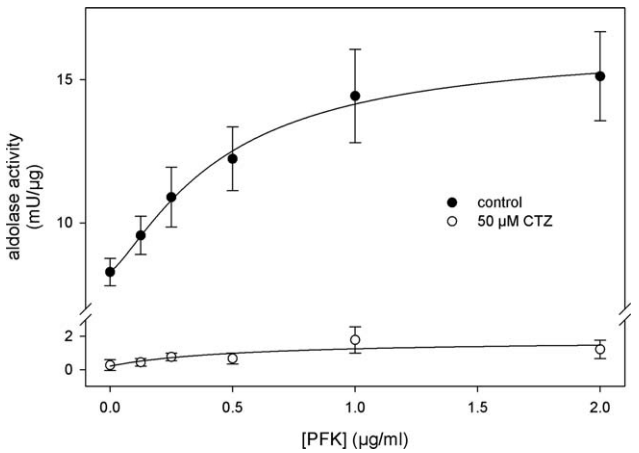

Figure 7. Effects of PFK on aldolase activity in the absence and presence of 50 μM CTZ. Aldolase activity in the presence of 1 μg/mL aldolase and the concentrations of PFK indicated on the abscissa was assessed as described in the "Materials and Methods" section. Experiments were performed in the absence (filled circles) and in the presence of 50 μM CTZ (empty circles). Plotted values are mean $\pm$ standard errors of at least four independent experiments ($n = 4$). Solid lines were obtained fitting Eq. (7) to the plotted data.

The kinetic parameters for the modulation of aldolase by its substrate F1,6BP were evaluated in the absence and in the presence of CTZ and PFK. CTZ inhibited aldolase in the presence

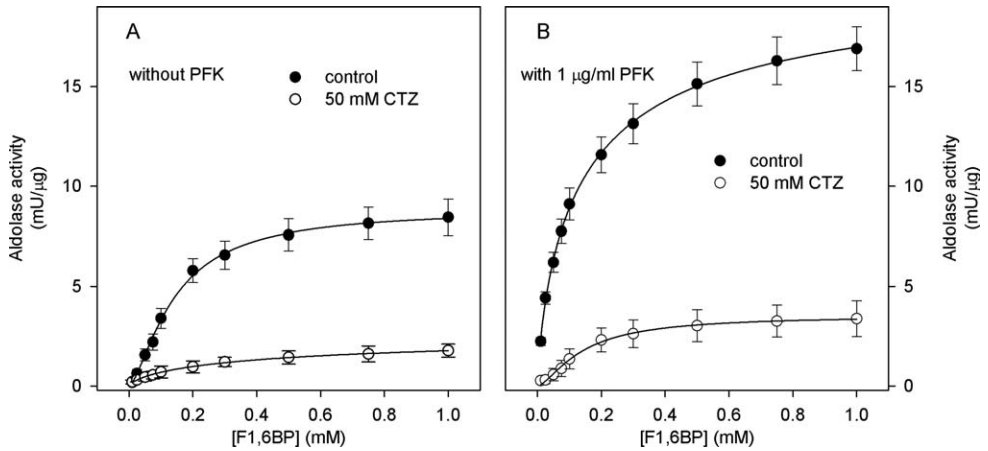

Figure 8. Effects of PFK and CTZ on the modulation of aldolase by its substrate. Aldolase activity was assessed as described under Materials and Methods in the absence and in the presence of 50 μM CTZ, as indicated. Panels A: control experiments in the absence of PFK. Panels B: aldolase activity in the presence of 1 μg/mL PFK. Plotted values as mean $\pm$ standard error of four independent experiments. Solid lines are the results of the adjust of Eq. (6) to the experimental plotted data.

Table 2
Kinetic parameters for aldolase modulation by F1,6BP

	Control	50 μM CTZ	1 μg/mL PFK	1 μg/mL PFK + 50 μM CTZ
V_{max} (mU/μg)	8.7 $\pm$ 0.9	1.6 $\pm$ 0.2[a]	17.9 $\pm$ 1.8[a]	4.1 $\pm$ 0.5[b,c]
$K_{0.5}$ (μM)	68.1 $\pm$ 4.2	103.4 $\pm$ 9.7[a]	34.1 $\pm$ 3.8[a]	89.1 $\pm$ 3.9[b,c]
n	1.4 $\pm$ 0.2	2.0 $\pm$ 0.2[a]	0.9 $\pm$ 0.3[a]	2.1 $\pm$ 0.2[b,c]

The parameters were calculated as described under "Materials and Methods" section.
[a] $P < 0.05$ compared to control (Student's t-test).
[b] $P < 0.05$ compared to 50 μM CTZ.
[c] $P < 0.05$ compared to 1 μg/mL aldolase.

of all concentrations of F1,6BP tested, in the absence and in the presence of PFK (Fig. 8). This effect resulted in a decrease of the enzyme maximal velocity and affinity for its substrate (Table 2). On the other hand, PFK promoted an increase in both the aldolase maximal velocity and the affinity of F1,6BP. Taking another point of view, the effects of CTZ on aldolase in the presence of PFK are attenuated, when compared to the effects of the drug in the absence of the kinase (Table 2). These results do not suggest that PFK protects aldolase against CTZ inhibition, but clearly demonstrate that the interaction between the two enzymes upregulates aldolase increasing both, its maximal velocity and its affinity for the substrate.

DISCUSSION

CTZ has been described as a potential antineoplastic drug (*20, 23, 27, 28, 37, 38*). Its antineoplastic properties are associated with its ability to decrease glucose consumption and energy metabolism in tumor cells (*27, 28*). There are several proposed targets for the action of CTZ on cell metabolism, and many of these proposed targets are involved in the glycolytic pathway (*38, 39*). We have previously demonstrated that this drug decreases the viability of breast cancer cells by inhibiting the glycolytic pathway (*20*) and that this inhibition is probably due to a direct effect of CTZ on PFK (*23, 29*). The data presented here add novel information concerning the ability of CTZ to affect glycolytic enzymes and, therefore, cancer cell metabolism. It appears that aldolase is able to prevent the inhibitory effect of CTZ on the major glycolytic regulatory enzyme, PFK. However, CTZ inhibits aldolase to a greater degree (98% inhibition for aldolase vs. 70% for PFK) and more efficiently ($I_{0.5}$ = 11 $\pm$ 1 μM and 28 $\pm$ 2 μM for aldolase and PFK, respectively). This means that, despite the protection against CTZ-induced PFK inhibition promoted by aldolase, CTZ still decreases the glycolytic flux through inhibition of the aldolase itself, the next enzyme in the pathway following PFK.

The protection against the inhibitory effects of CTZ on PFK promoted by aldolase would be expected. Because CTZ induces the dissociation of active PFK tetramers into inactive dimers (*23, 29*), aldolase, which binds to PFK dimers and renders them active (*10*), should counteract the effects of CTZ on PFK. However, another inhibitory modulator of PFK, citrate, disrupts the

protection conferred by aldolase against CTZ-induced inhibition of PFK. In the presence of citrate, CTZ is a more effective inhibitor of PFK than in the absence of the metabolite. In tumor cells, cytosolic citrate levels are elevated because of the need for fatty acid and cholesterol biosynthesis (*40*). Thus, CTZ might inhibit PFK in these cells due to the high levels of citrate.

An intriguing finding in the present work is the stimulatory property of PFK over aldolase. The ability of these enzymes to associate generating diverse heterooligomeric structures has been reported (*10*). However, the ability of PFK to increase aldolase activity has not been hitherto published. The fact that PFK augments the affinity of aldolase for its substrate strongly supports the hypothesis that the kinase, once associated to aldolase, stimulates its activity. This is an evidence for the channeling of the glycolytic metabolism, as have been previously proposed (*13–15*). Actually, this is not a classical channeling since PFK-bound aldolase is able to bind F1,6BP directly from the medium and not necessarily those formed by PFK catalysis. Nonetheless, the association of PFK and aldolase confers a catalytical advantage for both enzymes, stabilizing the active dimeric conformation of the former and stimulating the later.

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