Subunit interaction in mammalian aldolases

Jurgen SYGUSCH*‡ and Danielle BEAUDRY†

*Département de Biochimie, Faculté de Médecine, Université de Montréal, CP 6128, Station Centre-Ville Montréal, Québec, H3C 3J7 Canada and †Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, J1H 5N4 Canada

Enzyme inactivation was utilized to study subunit interaction in the homotetrameric glycolytic enzyme, aldolase. Isoenzymes from rabbit liver and skeletal muscle were inactivated in the presence of P_i and D-glyceraldehyde-*P* to a maximum stoichiometry of one modification per aldolase subunit. Subunit modification increased net negative charge on each subunit surface and was used to resolve modified aldolase isoenzymes into various chromatographic species. A combination of anion- (Mono Q) and cation- (Mono S) exchange chromatography

separated the modified aldolase homotetramers into three distinct enzyme populations: unchanged enzyme, fully modified enzyme corresponding to one ligand molecule incorporated per subunit and partially modified enzyme in which only one subunit out of four is modified. Both fully and partially modified species were devoid of catalytic activity. Activity loss through modification of a single subunit in both aldolase isoenzymes indicates tightly coupled communication between subunit active sites and suggests simple functional regulation of aldolases.

INTRODUCTION

The small molecules, D-glyceraldehyde-*P* and P_i , which covalently inactivate aldolase isoenzymes [1,2], were utilized to investigate subunit communication in mammalian aldolases. Aldolase reversibly cleaves fructose 1,6-bisphosphate into triose phosphates, D-glyceraldehyde-3-*P* and dihydroxyacetone-*P*. The enzyme is a homo-oligomer consisting of four subunits. To date communication between aldolase subunits has not been convincingly demonstrated. Evidence from peptide-binding assays [3] as well as antibody-binding studies [4] has alluded to the possibility of subunit communication, since interaction with a single aldolase subunit appeared to inhibit catalytic activity and peptide binding in the remaining subunits. The evidence was, however, not unequivocal as steric hindrance caused by large antibody size or the very low ionic strength used in the peptide-binding assay could have compromised subunit communication through loss of structural integrity.

In the present study, subunit interaction in the aldolase homotetramer was probed by analysing the activity of partially modified oligomeric enzyme populations. If aldolase subunits do not interact, then modification of a subset of subunits should affect only the activity of those subunits that have been modified. Subunit modifications, such as covalent addition of charged ligand molecules that modify the chromatographic properties of an enzyme are particularly advantageous in this approach. The addition of surface charges as a result of subunit modification can be exploited by ion-exchange chromatography to separate the modified enzyme populations. Complete loss of catalytic activity in a partially modified enzyme population would implicate subunit interaction and suggest subunit co-operativity.

Partially inactivated mammalian aldolases were separated by ion-exchange chromatography into distinct enzyme populations and assayed for activity. Surprisingly, modification of a single aldolase subunit was sufficient to abolish the catalytic activity of all the subunits.

EXPERIMENTAL

P_i was of analytical grade and was purchased as the sodium and potassium salts. Fructose 1,6-bisphosphate was purchased as the trisodium salt from Sigma Chemical Co. and utilized directly.

Dihydroxyacetone-*P* was bought as the dimethyl ketal form and deprotected according to the supplier's protocol (Sigma Chemical Co.). D-Glyceraldehyde-3-*P* was also purchased from Sigma Chemical Co. as the barium salt of the diethyl ketal form and prepared as described [5]. The deprotected triose phosphates were assayed enzymically and found to be more than 95% pure. NADH was supplied by Calbiochem. Other reagents and chemicals were all at least of reagent grade.

Enzymes

Fructose 1,6-bisphosphate aldolase was prepared by affinity elution ion-exchange chromatography [6] from rabbit skeletal muscle and rabbit liver, or the muscle isoenzyme was purchased directly from Boehringer-Mannheim. The specific activity of skeletal muscle and liver aldolase is 9–10 units/mg and 1.4 units/mg respectively, where 1 unit of activity represents 1 μ mol of fructose 1,6-bisphosphate cleaved/min at 22 °C. The molar concentration of rabbit skeletal muscle and rabbit liver aldolase was calculated by assuming an M_r of 158000 and specific absorption coefficients at 280 nm of 0.91 litre $\cdot$ g⁻¹ $\cdot$ cm⁻¹ [7] for muscle aldolase and 0.84 litre $\cdot$ g⁻¹ $\cdot$ cm⁻¹ for liver aldolase [8].

Aldolase activity was determined by following NADH oxidation spectrophotometrically at 340 nm using the triose phosphate isomerase- α -glycerolphosphate dehydrogenase-coupled assay as previously described [9].

The protein concentration of aldolase was determined by employing the BCA protein reagent assay available from Pierce Chemical Co. Protein phosphate content was analysed in triplicate by the procedure of Ames [10]. Incorporation of D-glyceraldehyde-*P* by aldolase isoenzymes was analysed in triplicate on the basis of phosphate content. Typical determinations of phosphate content utilized approx. 1 mg of modified aldolase and could reproducibly distinguish on a molar basis the incorporation of a single phosphate or glyceraldehyde-*P* moiety by one aldolase molecule. Estimated errors in stoichiometry of ligand incorporated by an aldolase tetramer were 10% or less.

Escherichia coli alkaline phosphatase was obtained from Pharmacia-LKB. One unit of phosphatase activity hydrolyses 1 μ mol of *p*-nitrophenol phosphate/min at 25 °C, pH 8.0.

‡ To whom correspondence should be addressed.

Radioactivity

Carrier-free acid-free ^{32}P -labelled P_i (10 mCi/ml) was purchased from Amersham Radiochemical Corporation. Radiolabelled samples were dissolved in Ready Safe (Beckman) and counted with a Beckman liquid scintillation counter LS8000. Unincorporated radioactivity was removed from the protein samples by dialysis until no further changes in radioactive counts could be detected between successive buffer changes. All radioactive counts were corrected to reference day 0 in the case of P_i labelling. In labelling experiments, the labelled phosphate was diluted with unlabelled phosphate to a final specific radioactivity of 0.2 mCi/mmol.

P_i and D-glyceraldehyde-*P* inactivation kinetics

Muscle or liver aldolase in 100 mM Tris/HCl (pH 8.2)/1 mM EDTA was inactivated by incubation in the presence of various concentrations of P_i or D-glyceraldehyde-*P*. To stop the reaction after a predetermined period, the pH was adjusted to 7.4 with excess Tris/HCl buffer (1.5 M, pH 7.4). Reaction volumes were adjusted such that, at low P_i or D-glyceraldehyde-*P* concentrations, the concentration of unchanged substrate was in at least 20-fold excess over protein concentration. Unchanged phosphate or D-glyceraldehyde-*P* was removed by extensive dialysis of the protein sample against 100 mM Tris/HCl (pH 7.4)/1 mM EDTA (stopping buffer).

The ligand concentration ranged between 1 μM and 10 mM. The inactivation protocol varied slightly depending on inactivator concentration. To determine activity loss at high concentrations, residual catalytic activity was assayed every 2 h over a 6 h period, at intermediate concentrations (10–200 μM), residual activity was measured at least several times over a 24 h period, and, at very low concentrations (5 μM or less), activity loss was only apparent after a 48 h incubation period. In the absence of inactivator, controls displayed no loss of catalytic activity.

Chromatography of inactivated aldolase isoenzymes

Muscle or liver aldolase (0.2 mg/ml) incubated in the presence of P_i or D-glyceraldehyde-*P* was applied to an FPLC Mono Q (HR 5/5) ion exchanger (Pharmacia) equilibrated with 20 mM Bistris, pH 6.5. The Mono Q column was washed with three column volumes of buffer and eluates were pooled for subsequent characterization. The application of a 1 M NaCl linear salt gradient, at flow rate of 1 ml/min, released a second fraction from the anion exchanger. This fraction was dialysed and applied to an FPLC Mono S (HR 5/5) cation exchanger (Pharmacia) equilibrated with 10 mM Tes (potassium salt), pH 7.4. The Mono S column was washed with 3 vol. of buffer. The application of a 1 M NaCl linear salt gradient at flow rate of 1 ml/min released the remaining adsorbed fraction. Both the flow-through fractions and the fractions released by the salt gradient were pooled for further characterization. A_{280} measurement was used to detect protein.

Dephosphorylation

Autophosphorylated aldolase (33 μM ; 1 ml) was incubated with alkaline phosphatase (0.02 unit) in 100 mM Tris/HCl (pH 7.8)/1 mM Mg Cl_2 buffer. Aliquots were removed at various times for analysis of phosphate content and residual activity. To stop each dephosphorylation reaction precisely, 100 mM EDTA was added to each aliquot to a final concentration of 10 mM.

Re-activation

Aldolases inactivated in the presence of D-glyceraldehyde-*P* were fractionated by ion-exchange chromatography as described above. Modified enzyme (1 mg/ml) was incubated in the presence of 1 mM dihydroxyacetone-*P* for 16 h in 100 mM triethanolamine/HCl, pH 7.4, and assayed for residual activity.

RESULTS

Inactivation kinetics

Kinetics describing activity loss in the presence of phosphate and D-glyceraldehyde-*P* were determined for both aldolase isoenzymes at room temperature (22 °C), pH 8.2, by assaying residual catalytic activity at regular intervals. At pH values below 8.0, inactivation could not be detected at room temperature, even after overnight incubation. Activity loss was first-order with time, demonstrated saturation behaviour and, on the basis of double-reciprocal plots, was characterized in terms of two kinetic parameters describing maximal rate of activity loss (V_m) and reversible ligand binding (K_m) [11]. The kinetic parameters are shown in Table 1. The linear correlation coefficients describing the agreement of each analysis with the inactivation data were better than 0.99. Double-reciprocal plots displayed no breaks of deviation from linearity over the entire concentration range. From the results shown in Table 1, inactivation of the isoenzymes in the presence of P_i and D-glyceraldehyde-*P* is very similar.

Maximal inactivation of the aldolase isoenzymes by either P_i or D-glyceraldehyde-*P* was stoichiometric and corresponded to incorporation of one phosphate moiety per aldolase subunit.

Chromatography of ligand-modified aldolases

Incorporation of a phosphate moiety at near-physiological pH increases the negative charge on the protein, and was used as the basis for separation of modified from unmodified isoenzyme by anion-exchange chromatography. At pH 6.5, two distinct fractions were detected by measurement of A_{280} ; a representative chromatograph is shown in Figure 1. Fraction 1, not retained by the anion-exchange column, corresponded to fully active enzyme. Analysis of its phosphate content revealed it to be unmodified enzyme. Fraction 2, which was eluted from the column by the NaCl gradient at 0.1 M, represents modified enzyme on the basis of phosphate content. Modified isoenzymes were devoid of catalytic activity. No difference in elution pattern was detected whether ligand was incorporated once per tetramer or once per subunit.

Fractionation of the multiply modified enzyme population was carried out by cation-exchange chromatography. Results from the previous chromatography indicated that retention of

Table 1 Limiting rate of activity loss and binding constants describing inactivation kinetics of aldolase isoenzymes in the presence of P_i or D-glyceraldehyde-*P*

Ligand inactivator	Muscle		Liver	
	$10^{-3} \times V_m$ (min^{-1})	K_m (mM)	$10^{-3} \times V_m$ (min^{-1})	K_m (mM)
P_i	3.18	0.77	3.91	0.67
D-Glyceraldehyde- <i>P</i>	4.78	0.86	4.26	0.70

Figure 1 Elution profile of partially inactivated aldolase isoenzymes by anion-change chromatography

Muscle aldolase allowed to react in the presence of P_i was applied to a Mono Q ion exchanger equilibrated with 20 mM Bistris, pH 6.5. Protein was detected by A_{280} (—). Fraction 1 corresponds to native enzyme. Application of a salt gradient of 1 M NaCl (---) releases fraction 2 from the anion exchanger. Fraction 2 corresponds to modified material and is devoid of catalytic activity. A similar elution pattern was observed for muscle aldolase inactivated in the presence of D-glyceraldehyde- P as well as liver aldolase inactivated in the presence of P_i or D-glyceraldehyde- P .

Figure 2 Cation-exchange chromatography of modified aldolase isoenzymes

Phosphorylated rabbit muscle aldolase corresponding to protein released by the salt gradient in Figure 1 (fraction 2) was dialysed and applied to a Mono S cation exchanger equilibrated with 10 mM Tes (potassium salt), pH 7.4. Protein was detected by A_{280} (—). Fractions 1 corresponds to fully modified muscle aldolase and is not retained by the column. Fraction 2, which is released by the 1 M NaCl salt gradient (---), corresponds to partially modified muscle aldolase. The same elution pattern was observed for reaction of muscle aldolase with D-glyceraldehyde- P and liver isoenzyme with P_i or D-glyceraldehyde- P .

aldolase was restricted under appropriate conditions to interaction by the stationary phase with only a very limited region on a single aldolase subunit. At an appropriate pH therefore, a subunit modified by phosphate ion or D-glyceraldehyde- P should not interact with a cation exchanger, whereas unmodified subunits should retain their capacity to interact with the cation exchanger. This rationale was used to separate partially modified from fully modified tetramer molecules.

At pH 7.4, the inactivated isoenzyme population was separated into two distinct fractions by cation-exchange chromatography; a representative chromatograph is shown in Figure 2. Fraction 1 was not retained by the cation exchanger and on the basis of phosphate content corresponds to fully modified protein, with

Figure 3 $[^{32}P] P_i$ incorporation by singly modified aldolase isoenzymes at various times

$[^{32}P] P_i$ uptake (a) muscle and (b) liver aldolase isoenzyme with one subunit previously phosphorylated was measured. The reaction conditions were 5 mg of rabbit muscle or liver enzyme in 1 ml of 100 mM Tris/HCl buffer (pH 8.2)/1 mM EDTA with 10 mM P_i final concentration (specific radioactivity 0.2 mCi/mmol). The reaction was stopped by the addition of 1 ml of 1.5 M Tris/HCl, pH 7.4.

one ligand incorporated per subunit. Fraction 2, which was eluted at 0.2 M NaCl, represents modified enzyme having only a single phosphate moiety per tetramer. Variation in stoichiometry in repeated analyses was less than 0.1 mol of phosphate incorporated per mol of tetrameric enzyme. At pH values below 8.0, singly and fully modified aldolase isoenzymes remained stable, in terms of incorporated phosphate and devoid of catalytic activity even after prolonged storage as precipitates in 80 % saturated $(NH_4)_2SO_4$.

Enzyme populations

Ion-exchange chromatography of aldolase isoenzymes incubated with P_i or D-glyceraldehyde- P , at concentrations $\ll K_m$, showed fully modified isoenzyme populations prevalent only at later incubation times whereas, at saturating concentrations, singly modified enzyme species were detectable only during the initial phase of inactivation. The specific activity of unmodified enzyme populations did not change from the initial value at the beginning of each incubation period, consistent with modified isoenzyme, both singly and fully, being devoid of catalytic activity. Stoichiometries that would indicate populations or mixtures of populations other than those corresponding to singly or fully modified aldolase tetramers were not observed using the phosphate assay.

Labelling

Singly phosphorylated aldolase isoenzymes were labelled in the presence of radioactive P_i . Label incorporated as a function of time exhibited a 'burst' phenomenon (Figure 3). Separation of labelled species by cation-exchange chromatography was consistent with radioactivity incorporated only into fully modified tetramer fractions. To ensure that label corresponded to phos-

Figure 4 Enzyme populations obtained by ion-exchange chromatography of fully phosphorylated aldolase isoenzymes that had been dephosphorylated in the presence of alkaline phosphatase for various lengths of various time

The three enzyme populations correspond on the basis of stoichiometry to fully phosphorylated aldolase isoenzyme (▲), partially phosphorylated isoenzyme (■) and active unmodified isoenzyme (●). (a) muscle aldolase; (b) liver aldolase.

phate incorporated covalently into fully modified tetramer, the labelled tetramer was rechromatographed using a Mono Q anion exchanger. Unincorporated P_i , which is eluted from the Mono Q column after and distinct from modified tetramer molecules, was not detected. The stoichiometry of the fully modified tetramer molecules corresponded, on the basis of incorporated radiolabel, to four phosphate ligands per aldolase tetramer. Stoichiometries corresponding to phosphorylation of less than fully modified tetramer were not observed.

Dephosphorylation

To eliminate the possibility of minor species of doubly or triply phosphorylated intermediates (A_2 or A_3) being coeluted with the fully modified tetramer fraction, the chromatographic behaviour of fully phosphorylated isoenzymes that had been partially dephosphorylated at pH 7.8 by alkaline phosphatase was examined. The ion-exchange chromatographic procedure used to isolate the various muscle and liver isoenzyme populations yielded three distinct products of the dephosphorylation reaction (Figure 4). The three fractions corresponded to unmodified, fully modified A_4 tetramer and an intermediate species that chromatographed as a single entity. On the basis of the stoichiometry shown in Table 2, the identity of the intermediate species is consistent with the existence of triply phosphorylated tetramer molecules in the initial stages of dephosphorylation and singly phosphorylated tetramer molecules in the final stages. The presence of doubly phosphorylated tetramer molecules at intermediate incubation times, although not inconsistent with the stoichiometry data, can only be inferred. Generation of mixtures

Table 2 Stoichiometries of partially dephosphorylated aldolase isoenzyme populations resolved by ion-exchange chromatography

Values in parentheses were determined after incubation of the fraction overnight (16 h) at pH 7.4 in the presence of 10 mM phosphate and rechromatographed. ND, Not determined; NS, species not detectable by chromatography.

Time (min)	Stoichiometry (mol of P_i /mol of enzyme)	
	Muscle	Liver
0.0	4.0	4.0
0.5	2.8	2.8
1.0	2.6 (2.6)	2.5 (2.5)
2.0	2.2	2.1
3.0	2.0	1.8
4.0	ND	1.5 (1.5)
5.0	1.5 (1.5)	1.3
10.0	1.00	NS

of A_1 and A_3 species only would require the A_2 species to be intrinsically more labile than the A_1 , A_3 and A_4 species. However, the stoichiometries of the dephosphorylation reaction mixtures and those of the same reaction mixtures rapidly quenched with 50% (v/v) cold acetone did not differ within experimental error, suggesting that the A_2 species is not unduly labile. Minor species of doubly and triply phosphorylated tetramer molecules, if generated during inactivation, must thus behave chromatographically identically with singly modified tetramer molecules and not with fully modified tetramer molecules, and would have been detected by the radiolabelling studies.

To rule out further the possibility that partially modified tetramer molecules are labile and rapidly converted into the fully modified tetramer molecules even at pH 7.4, the pH used to stop the phosphorylation reaction, the partially dephosphorylated species were incubated at pH 7.4 overnight in the presence of P_i (10 mM). Chromatography revealed no additional species corresponding to either fully modified or even fully dephosphorylated tetramer molecules. The stoichiometries of the incubated intermediate species were unchanged within experimental error with respect to their preincubation values.

Re-activation

To investigate product interaction with the inactivated isoenzymes, isoenzymes that had previously been incubated with D-glyceraldehyde- P were incubated in the presence of 1 mM dihydroxyacetone- P . Complete restoration of catalytic activity was obtained for both isoenzymes after overnight incubation at pH 7.4, and this was independent of the number of subunits modified. At pH 7.4, modification does not take place and controls (from which dihydroxyacetone- P was omitted) did not recover activity after overnight incubation.

DISCUSSION

Distinct populations corresponding to aldolase isoenzymes possessing partially modified subunits were purified by ion-exchange chromatography. Mono Q anion-exchange chromatography separates, near physiological pH, unchanged rabbit skeletal muscle and liver aldolase from isoenzymes that have incorporated a net negative charge (Figure 1). The modified isoenzyme populations can be further fractionated by Mono S cation-exchange chroma-

tography into two populations of fully and partially modified enzymes (Figure 2). On the basis of phosphate content, the partially modified isoenzyme population corresponds within experimental error to one subunit modified per aldolase tetramer. Chromatography of modified isoenzyme populations is independent of aldolase isoenzyme and ligand inactivator and implies interaction at the molecular level of only a limited region of a modified subunit with the stationary phase. Substrate affinity elution chromatography [12] used to purify aldolase isoenzymes supports the interpretation of limited interaction of each isoenzyme with the stationary phase. Fructose 1,6-bisphosphate bound to the aldolase active site augments the net negative charge by a maximum of 4 units per subunit and dissociates the isoenzyme from the CM-Sepharose stationary phase. The superior resolution of Mono Q and Mono S has enabled the discrimination of a singly modified enzyme population that corresponds to a net increase in negative charge of 2 units. Stoichiometries corresponding to two or three modified subunits were not observed over the concentration ranges examined.

Covalent modification of a single aldolase subunit was sufficient to abolish completely expression of catalytic activity in all aldolase subunits. Inactivation was observed in the presence of both phosphate ion and D-glyceraldehyde-*P* and was independent of the isoenzyme species. Loss of catalytic activity from both isoenzymes can be described by a first-order process with no kinetic evidence for more than one enzyme species. Maximal rates of activity loss and K_m values, shown in Table 1, are very similar for both ligand inactivators and aldolase isoenzyme species, consistent with an activation process, possibly conformational in origin given the large intrinsic differences in reactivity between P_i and aldehydes.

Controls shown in Table 2 suggest that the doubly or triply modified isoenzyme intermediates, A_2 and A_3 respectively, would be co-eluted with the singly modified species A_1 and this would be reflected by higher stoichiometries. The possibility that A_2 and A_3 intermediates are present during inactivation at only low concentration relative to the A_1 species and not detected by the phosphate assay was ruled out by radiolabelling studies that are capable of detecting intermediates at subnanomolar concentrations. Labelling each isoenzyme A_1 species with [^{32}P] P_i (Figures 3(a) and 3(b)) shows apparent burst kinetics initially with respect to ligand uptake, after which the rate of label incorporation becomes linear. Enzyme populations other than fully labelled phosphorylated species were not detected even when labelling coincided with apparent pre-steady-state conditions.

Our conclusions corroborate previous findings of functional interaction between aldolase subunits. Binding studies using erythrocyte band 3 peptides have been interpreted on the basis of a single band 3 peptide binding to one aldolase subunit [3], which reduces the affinity of the remaining subunits. In antibody inhibition studies specific non-cross-reacting antibodies directed against homomeric aldolases inhibited the activity of heteromeric aldolase hybrids possessing only one subunit recognized by the antibody [4]. Although binding modified the functional behaviour of the aldolase subunits in each case, these studies could not unequivocally rule out alternative interpretations such as steric hindrance caused by large antibody size or low ionic strength used in the peptide-binding assay destabilizing structural integrity resulting in compromised activity or binding.

Covalent modification studies using small ligand inactivators at near-physiological ionic strength are conceptually simpler to interpret than peptide- and antibody-binding assays that depend on larger macromolecules. Modification by P_i results in phosphorylation of a specific C-terminal residue in each aldolase

isoenzyme, and dephosphorylation restores full catalytic activity [2] consistent with maintenance of structural integrity. Stoichiometric attachment of D-glyceraldehyde-*P* at homologous subunit loci is consistent with the recovery of full catalytic activity by incubation with the co-substrate dihydroxyacetone-*P* and also implies maintenance of structural integrity. The loss of catalytic activity caused by specific modification of a single aldolase subunit therefore unequivocally demonstrates the existence of subunit communication between functional aldolase protomers.

Not many oligomeric enzymes have been shown to display communication among their subunits. Several oligomeric enzymes that were not previously known to exhibit allosteric behaviour have been shown to demonstrate subunit-co-operativity after chemical modification [13], when modified by site-directed mutagenesis [14] or when assayed in the presence of inhibitors [15–17]. The experimental methodology described should be amenable to the study of subunit co-operativity in any oligomeric protein. Our approach requires only that the ligand inactivator possesses a net charge so that the modified oligomeric protein can be selectively purified by ion-exchange or electrofocusing chromatography. A similar approach exploiting differential behaviour in dye-ligand chromatography was used to resolve a hybrid complex of the multifunctional heterotetrameric enzyme anthranilate synthase-phosphoribosyltransferase [18]. The kinetic properties of the hybrid complex demonstrated that binding of a single inhibitor molecule to one subunit was sufficient to propagate a conformational change that affected the active site of a companion subunit.

It has been proposed that aldolase activity is controlled during glycolysis via adsorption/desorption from other cellular constituents [19]. Aldolase has been shown to be inhibited on binding to thin filament proteins in resting muscle [20]. When localized to the triad junction in skeletal muscle, the site of excitation-contraction coupling, aldolase activity is highly compromised [21]. Implicit in these studies was the assumption that aldolase attachment to these very large macromolecular structures involved only a single subunit. Our findings support the interpretation of binding to a single subunit abrogating catalytic activity in all subunits and suggest that regulation of aldolase activity may occur via interaction requiring only one subunit. Tight coupling among aldolase subunits as such would simplify considerably the recognition process, since aldolase receptors would not be required to bind all subunits in order to mediate aldolase activity. An intriguing conjecture is the possibility that catalysis in mammalian aldolases requires a tightly coupled or concerted quaternary conformational fluctuation involving all subunits rather than a conformational process taking place independently in each subunit, and as such may not be detected from steady-state kinetics.

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