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# Spontaneous subunit exchange in porcine liver fructose-1,6-bisphosphatase

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**Abstract No evidence to date suggests the possibility of subunit exchange between tetramers of mammalian fructose-1,6-bisphosphatase. An engineered fructose-1,6-bisphosphatase, with subunits of altered electrostatic charge, exhibits spontaneous subunit exchange with wild-type enzyme in the absence of ligands. The exchange process reaches equilibrium in approximately 5 h at 4°C, as monitored by non-denaturing gel electrophoresis and anion exchange chromatography. Active site ligands, such as fructose 6-phosphate, abolish subunit exchange at the level of the monomer, but permit dimer–dimer exchanges. AMP, alone or in the presence of active site ligands, abolishes all exchange processes. Exchange phenomena may play a role in the kinetic mechanism of allosteric regulation of fructose-1,6-bisphosphatase.** 

*Key words:* Gluconeogenesis; Protein assembly; Protein engineering; Mammalian enzyme; Glucose metabolism; Subunit interface

## 1. Introduction

Fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) catalyzes the hydrolysis of fructose 1,6-bisphosphate ($F16P_2$) to fructose 6-phosphate (F6P) and inorganic phosphate ($P_i$) [1–3]. The futile cycle defined by FBPase and fructose 6-phosphate 1-kinase is subject to strict hormone and metabolite regulation [4]. Inhibition of FBPase by AMP and $F26P_2$ is synergistic [5]. AMP binds cooperatively (Hill coefficient of 2), approximately 30 Å from the nearest active site [6], whereas $F26P_2$ binds at the active site [7].

FBPase in crystals is a tetramer of identical subunits ($M_r = 37\,000$), and exists in one of two conformational states called R and T. Subunits of the R-state enzyme, which crystallizes in the presence of active site ligands, lie approximately in a plane. By past convention [8], the subunits of the tetramer are named as in Fig. 1. A 17° rotation of the subunits C1–C2 with respect to subunits C3–C4 about a molecular twofold axis of symmetry transforms the R-state into the T-state conformer [9]. Complexes of FBPase with AMP in the presence of $F16P_2$, $F26P_2$ and F6P are all in the T-state [10–12], whereas in the absence of AMP, the crystalline enzyme is in the R-state [13–15].

Analytical ultracentrifugation, using protein or product absorbance for detection [16–18], indicates only FBPase tetramers, the one exception being a thermophilic FBPase dimer [19]. In addition, FBPase exhibits retention times in size exclusion chromatography which are consistent with a tetramer [20].

Subunit exchange is an alternative to analytical ultracentrifugation in monitoring the dissociation of oligomeric proteins into subunits [21–23]. An increasing number of proteins have been identified that undergo subunit exchange reactions. Hemoglobin, thymidylate synthetase, GroES, α-crystallin, smooth muscle myosin filaments, adenylosuccinate lyase, and adenylosuccinate synthetase exhibit subunit exchange in the absence of denaturants [24–30]. Apart from the above, most oligomeric proteins require denaturants, such as urea or extremes in pH, or the use of hydrostatic pressure to induce subunit exchange [31–33]. In some instances, most notably lactate dehydrogenase, hybrids between two isozymes form in vivo [34]. In order to probe whether subunit exchange occurs in FBPase, a mutant enzyme was engineered that differs from the wild-type enzyme in its electrostatic charge. Non-denaturing gel electrophoresis and anion exchange chromatography reveal spontaneous subunit exchange between the engineered and wild-type FBPases.

## 2. Materials and methods

Catalysis and AMP inhibition are sensitive to mutations near the N-terminus of recombinant porcine FBPase [35]. Hence, initial efforts focused on the addition of charged residues to the C-terminus of the polypeptide chain. C-terminal additions of 10 histidines or 10 glutamates, however, resulted in the expression of either unstable proteins or proteins that behaved poorly in chromatographic separations. The mutation of surface lysines at positions 268, 269 and 272 to glutamate provided a stable and easily purified FBPase. Specific base changes in double-stranded plasmid employed the Quickchange™ Mutagenesis kit (Stratagene). The procedure involves 20 cycles of linear PCR using two complementary mutagenic primers (listed below). The mixture is then treated with *Dpn*I restriction enzyme in order to remove parental DNA, leaving only mutated nicked circular DNA strands. The nicked DNA is then transformed into XL1-Blue competent cells for amplification of the mutant double-stranded plasmid DNA. Two sets of primers were used sequentially to produce the triple mutant. K268E and K269E were introduced using a forward primer with the sequence: 5′-GGGATCTTTATGTACCCAGCAAACGAGGAAAGCCCCGAAGGAAAGTTAAGACTGC-3′. The forward primer for the

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*Abbreviations:* FBPase, fructose-1,6-bisphosphatase; $F16P_2$, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; $P_i$, inorganic phosphate; $F26P_2$, fructose-2,6-bisphosphate; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis


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K272E mutant was: 5′-CCCAGCAAACAAGAAAAGCCCCGAAGGAAAGTTAAGACTGCTAT-3′. The reverse primers used in the mutagenesis reaction were the complement of the forward primers listed above. Mutations, and the integrity of the resulting gene, were confirmed by sequencing the promoter region and the entire open reading frame. The Iowa State University sequencing facility performed all DNA sequencing, using the fluorescent dye dideoxy terminator method.

Protein expression and purification were as described previously [36]. FBPase-deficient *Escherichia coli* were used for the expression of wild-type (recombinant porcine liver) and engineered (lysines 268, 269 and 272 to glutamate) FBPases. Protein purity and concentration throughout the purification procedure were monitored by SDS–PAGE [37] and Bradford assays [38], respectively.

Circular dichroism (CD) spectra of wild-type and engineered FBPases were collected at room temperature on a Jasco J710 CD spectrometer in a 1 cm cell, using a protein concentration of 0.30 mg/ml. Spectra were an average of three independent scans collected from 200 to 260 nm in increments of 1.3 nm. Each spectrum was blank-corrected using software provided with the instrument.

Assays for the determination of specific activity, $k_{cat}$, and activity ratios at pH 7.5 and 9.5 employed the coupling enzymes, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (Roche) [1]. The reduction of $NADP^+$ to NADPH was monitored by absorbance change at 340 nm. All other assays used the same coupling enzymes, but monitored NADPH production by its fluorescence emission at 470 nm with an excitation wavelength of 340 nm. All kinetic assays were performed at 22°C. Initial kinetic rates were analyzed by programs written in MINITAB, using an $\alpha$ value of 2.0 [39], or by the ENZFITTER software package [40]. Kinetic parameters and equations are provided in [36].

Non-denaturing PAGE was performed according to [37], but omitting the SDS and 2-mercaptoethanol from the loading and running buffers. In-gel activity assays were performed as in [41]. After electrophoresis, the gel was placed on a bed of solid 0.5% low melting agarose (total volume 20 ml), containing 5 mM MgCl, 150 mM KCl, 15 μM $NADP^+$, 1 mM FBP, 10 μg/ml phosphoglucose isomerase, 5 μg/ml glucose-6-phosphatase dehydrogenase, 0.6 μM nitroblue tetrazolium, and 1.6 μM phenazine methosulfate (all components added after agarose had cooled to 37°C). Color was allowed to develop at 22°C, which was then arrested after 10–15 min by the addition of 10% acetic acid.

Subunit exchange reactions monitored by non-denaturing PAGE were performed in HEPES buffer (pH 7.5) using equal protein concentrations (0.5 mg/ml) for each enzyme and a temperature of 4°C. Ligand concentrations in subunit exchange reactions are in the figure legends. Tetramer hybrids, analyzed by anion exchange chromatography, were produced from a mixture of wild-type and engineered FBPases, dialyzed overnight against 20 mM Tris–HCl (pH 8.3) at 4°C. The hybrid mixture was then loaded onto a DEAE HPLC column, and eluted by a gradient, 0–300 mM in NaCl. AMP was added immediately to the eluted hybrids, in order to arrest exchange prior to analysis by non-denaturing PAGE.

## 3. Results

Wild-type and engineered FBPases exhibit identical properties during purification except that, as anticipated, they elute at NaCl concentrations of 150 and 300 mM, respectively, from a DEAE-Sepharose column. The wild-type and mutant enzymes are at least 95% pure with no evidence of proteolysis on the basis of non-denaturing PAGE or SDS–PAGE. The CD spectra of wild-type and engineered enzymes, a measure of secondary structure, are identical to within experimental uncertainty. The $K_m$ for $F16P_2$ and $K_i$ for $F26P_2$ for the engineered enzyme are approximately four- and 12-fold greater than that of the wild-type enzyme (Table 1). All other kinetic parameters, as well as mechanisms of inhibition by AMP and $F26P_2$, are unchanged by the mutations.

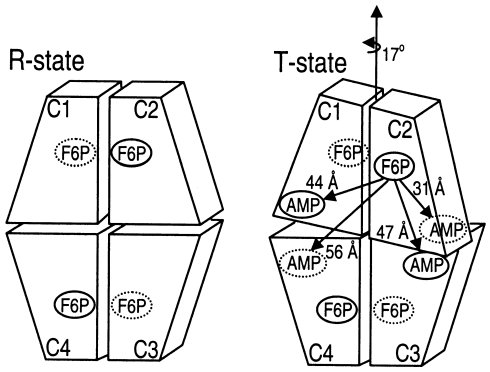


Fig. 1. Quaternary conformational states of FBPase. F6P and AMP identify the relative positions of active and allosteric inhibitor sites, respectively. Dashed outlines represent binding sites on the face of the tetramer hidden from view. Distances between the active site of subunit C2 and the four AMP binding sites of the tetramer are shown. A 17° rotation of the C1–C2 subunit pair, as shown, converts the T-state into the R-state conformer.

Subunit exchange between the wild-type and engineered proteins is apparent from non-denaturing gels. The expected pattern of five electrophoretically distinct bands represent wild-type:engineered subunit ratios of 4:0, 3:1, 2:2, 1:3, and 0:4. Subunit exchange is time-dependent, and exhibits first order kinetics (Fig. 2A,B). The lines in Fig. 2 represent single exponential fits to data acquired from band densities averaged over three gels. Rate constants for the disappearance of wild-type and engineered homotetramers and the appearance of the three hybrid species are in Table 2. Incubation in 8 M urea for 5 min, followed by a 50-fold dilution and ammonium sulfate precipitation (the procedure takes a total of 10 min), results in a pattern identical to that observed after 6 h in a buffer without urea (data not shown). In-gel activity assays indicate that the hybrid enzymes retain full activity (Fig. 2D). Additionally, the specific activity of the hybrid mixture is equal to that of the homotetrameric enzymes. Ligands that bind to the active site permit only dimer–dimer exchange (Fig. 2C), with a decreased rate constant for the

Table 1
Kinetic parameters for wild-type and engineered mutants of FBPase

| FBPase | pH ratio[a] | $k_{cat}^b$ ($s^{-1}$) | $K_m$ $F16P_2$ [c] (μM) | $K_a$ $Mg^{2+}$ [d] ($mM^2$) | Hill $Mg^{2+}$ | $K_i$ $F26P_2$ [c] (μM) | $IC_{50}$ AMP[d] (μM) |
|---|---|---|---|---|---|---|---|
| WT | 3.3 | 22 ± 1 | 1.8 ± 0.1 | 0.67 ± 0.04 | 1.9 ± 0.1 | 0.12 ± 0.01 | 1.61 ± 0.05 |
| Eng | 3.4 | 21 ± 2 | 6.6 ± 0.7 | 0.78 ± 0.06 | 2.1 ± 0.2 | 1.5 ± 0.1 | 2.9 ± 0.2 |

[a]Ratio of the specific activities at pH 7.5 and 9.5.
[b]Specific activity determined at saturating substrate concentrations divided by total protein concentration.
[c]Determined at a $Mg^{2+}$ concentration of 5 mM.
[d]Determined at the $K_a$ of $Mg^{2+}$ for each enzyme and a $F16P_2$ concentration of 20 μM and 100 μM for wild-type and engineered FBPases, respectively.

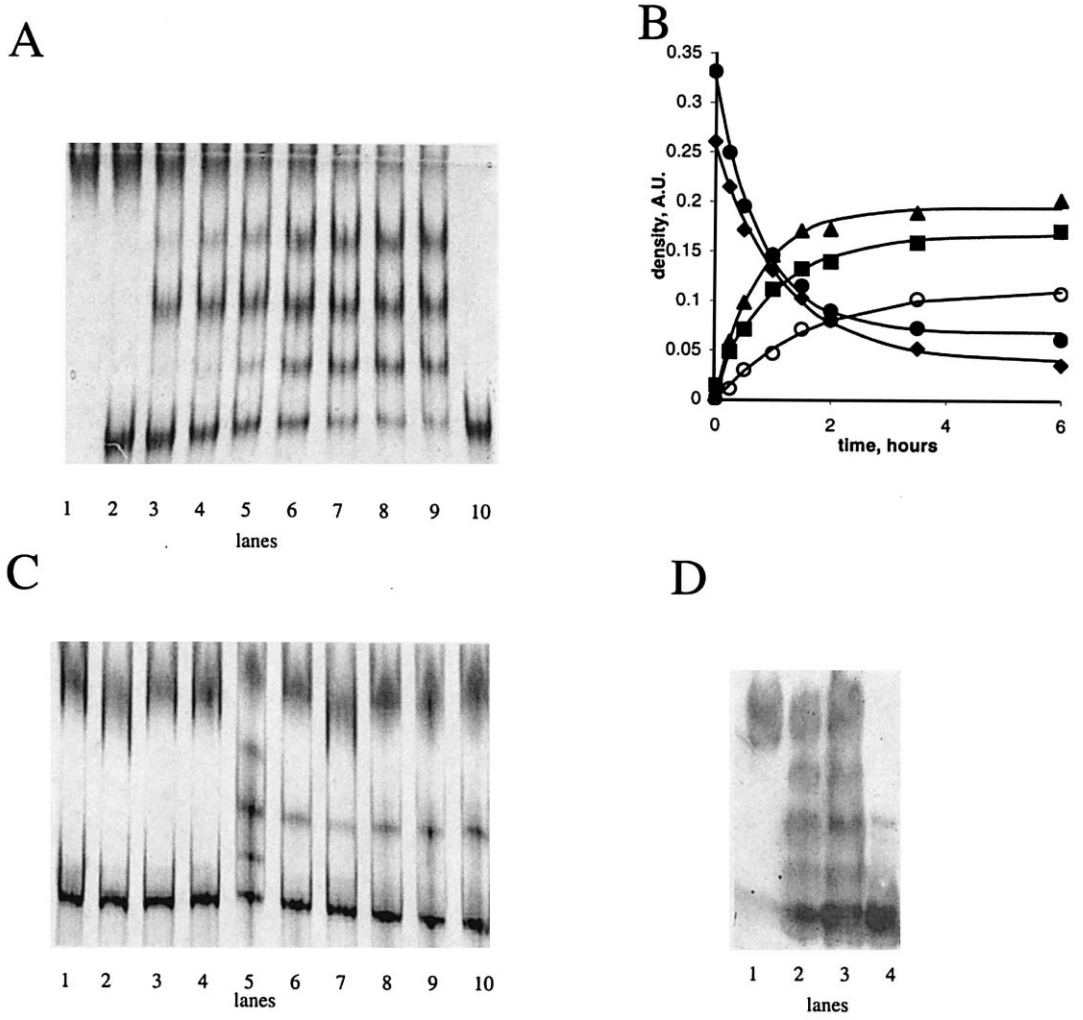


Fig. 2. Subunit exchange between wild-type and engineered FBPases. A: Time course of the exchange as seen by 6% non-denaturing-PAGE. Lanes 1 and 10 are purified wild-type and engineered FBPases, respectively. Lanes 2–9 are wild-type and engineered FBPases mixed at approximately equal concentrations (0.5 mg/ml) at the following times prior to loading the gel: 0, 0.25, 0.5, 1, 1.5, 2, 3.5, and 6 h, respectively. B: Time courses for the disappearance of homotetramers and the formation of hybrid tetramers, based on density scans of gels from A. The symbols represent (ratios listed are wild-type:3KE): (●) loss of wild-type homotetramer, (♦) loss of 3KE homotetramer, (▲) gain of 2:2 hybrid, (■) gain of 3:1 hybrid, and (○) gain of 1:3 hybrid. C: The effect of ligands on subunit exchange. Lane 1 contains wild-type and engineered enzymes mixed just prior to loading. Lanes 2–4 have the two FBPases along with AMP, AMP+F6P, and AMP+$F26P_2$, respectively, incubated for 12 h. Lane 5 has wild-type and engineered enzymes incubated for 6 h. Lanes 6–10 contain the two FBPases along with F6P+$KP_i$+$Mg^{2+}$, $F26P_2$, $F16P_2$, F6P+$KP_i$, and F6P, respectively, incubated for 12 h. Concentrations of AMP, $F16P_2$, $F26P_2$, F6P, $KP_i$, and $Mg^{2+}$ are 1, 0.5, 0.2, 5, 5, and 5 mM, respectively. D: In-gel activity assay of wild-type and engineered FBPases. Lane 1 has 4 μg of wild-type enzyme, lanes 2 and 3 have 7.5 μg and 15 μg of the hybrid mixture, respectively, and lane 4 contains 4 μg of engineered FBPase.

formation of the 2:2 hybrid relative to that obtained in the absence of ligands. No hybrids of any kind appear for up to 12 h in the presence of AMP, or in combinations of AMP with $F26P_2$ or F6P.

The hybrid tetramers possess different retention times on a DEAE anion exchange column, consistent with their mobilities on non-denaturing PAGE (Fig. 3). However, the appearance of six equally active peaks is unexpected. Non-denaturing PAGE analysis reveals that the peaks at 107 and 112 min are both 2:2 hybrids. Presumably, the spatial arrangement of subunits within the 2:2 hybrid affects its affinity for the DEAE column. Three 2:2 hybrids are possible, each differing with respect to distances between charged groups. Fushinobu and colleagues also observed partial resolution of different 2:2 hybrids in their purification of lactate dehydrogenase hybrids [42]. Purified hybrids revert to an equilibrium mixture in the absence of ligands, but the addition of AMP maintains purified hybrids as single entities. The specific activities of all isolated hybrids, assayed within 2 min of elution from the anion exchange column before the addition of AMP, are approximately equal to that of hybrid mixture.

Table 2
Rate constants for hybrid tetramer formation and homotetramer disappearance

| Subunit ratio WT:Eng[a] | Rate constant ($h^{-1}$) |
|---|---|
| 4:0 | 1.3 ± 0.1 |
| 3:1 | 0.85 ± 0.06 |
| 2:2 | 1.3 ± 0.1 |
| 1:3 | 0.63 ± 0.06 |
| 0:4 | 0.95 ± 0.05 |

[a]Ratio of wild-type to engineered subunits in the tetramer.

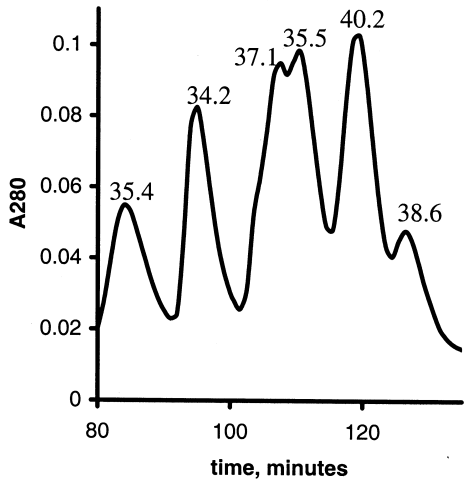


Fig. 3. Elution profile of a hybrid mixture from DEAE HPLC. The number above each peak indicates the specific activity assayed immediately after elution and before the addition of AMP.

## 4. Discussion

On the basis of steady-state kinetics the replacement of lysine at positions 268, 269, and 272 with glutamate modestly perturbs the affinities of $F16P_2$ and $F26P_2$. The elevated $K_m$ for $F16P_2$ and $K_i$ for $F26P_2$ in the engineered enzyme is probably due to a perturbation of Lys274 by the mutation of Lys272. The mutation of Lys274 to alanine results in a 20-fold increase in the $K_m$ of $F16P_2$ and a 1000-fold increase in the $K_i$ of $F26P_2$ [43]. The mutations above may alter as well the exchange properties of the engineered protein. Even so, any observation of subunit exchange here is possible only if both the wild-type and engineered FBPases participate. A non-exchanging, wild-type FBPase would block the formation of hybrid tetramers.

Subunit exchange can demonstrate the existence of dissociated subunits in equilibrium with an oligomeric protein, even when concentrations of dissociated subunits are vanishingly small or are in rapid exchange with the parent oligomer [21]. Data of Fig. 2 are compelling evidence in support of subunit dissociation/reassociation phenomena in FBPase. In the absence of ligands, FBPase must be in rapid equilibrium with a pool of monomers or a mixture of monomers and dimers. During the time course of subunit exchange in the absence of ligands, the 2:2 hybrids appear first, followed by the 1:3 and 3:1 hybrids.

Active site ligands selectively arrest the exchange phenomenon at the level of individual subunits. After a time sufficient to achieve an equilibrium mixture of hybrids, only the 2:2 hybrid forms in the presence of F6P, $F16P_2$ or $F26P_2$. By binding to residues from both the C1 and C2 subunits, the 6-phosphoryl group of active site ligands probably retards the dissociation of C1–C2 dimers into monomers.

AMP prevents subunit exchange altogether. As AMP binds exclusively to one subunit, it cannot be involved directly in a subunit cross-linking phenomenon. The number of intersubunit contacts between subunits C1 and C4 in the T-state conformer is greater than in the R-state. Thus, the stabilization of the C1–C4 interface (dimer–dimer interface) may impede subunit exchange. The simplest model that accounts for the exchange phenomena reported here is an ordered dissociation of subunits in which the enzyme must first be in the R-state before it can dissociate into C1–C2 dimers, followed by dissociation of C1–C2 dimers into monomers (Fig. 4). The dimer in the model above is an obligatory intermediate in the exchange of individual subunits. The data here, however, cannot unambiguously define the kinetic pathway of exchange. The model above is merely a plausible explanation of observations reported here.

The physiological consequence of subunit exchange (even if it occurs in vivo) is unclear. Although mammals have two isozymes of FBPase, there is no direct evidence suggesting the co-expression of both isozymes within the same cell type. If indeed isozymes of FBPase are segregated, then exchange cannot result in the formation of hybrids in vivo. No one to our knowledge has reported the isolation of FBPase hybrids from a mammalian tissue. Nonetheless, the similar isoelectric points and masses of the two isozymes may obscure the detection of such hybrid tetramers.

As subunit exchange occurs fairly rapidly under non-denaturing conditions, subunit exchange could have a bearing on the dynamics of the R- to T-state conformational change. More specifically, how are steric barriers at the dimer–dimer

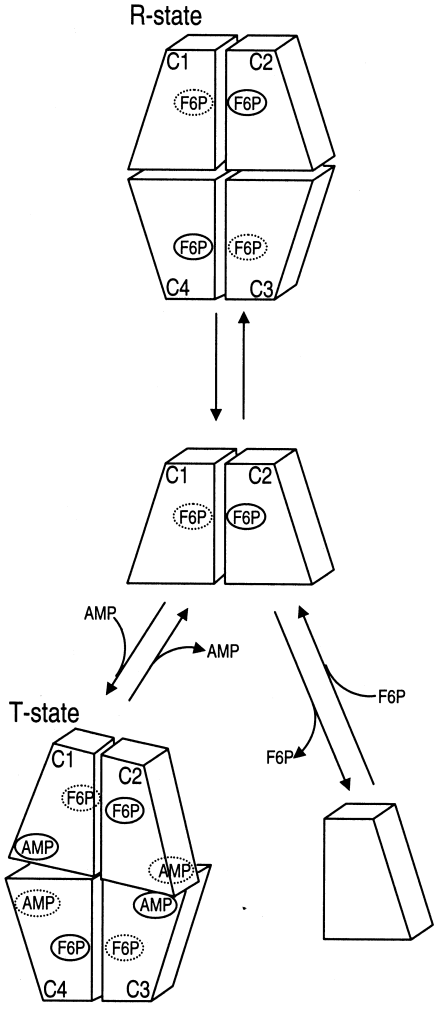


Fig. 4. Proposed pathway for subunit exchange and the R- to T-state transformations in FBPase. Subunit exchange and the quaternary conformational change in FBPase may both proceed through a dimer.

interface overcome during the transition between quaternary states? AMP may bind initially to an FBPase dimer (Fig. 4), altering its conformation so that reassociation results in a stable T-state tetramer. As AMP inhibition occurs rapidly, this model requires rapid dimer dissociation/reassociation. Rate constants for subunit exchange reported here do not exclude this possibility. The subunit exchange rate is not a measure of the rate of tetramer dissociation into dimers. The majority of dissociation events may result in immediate reassociation before dimer exchange occurs. Only occasionally will a subunit or dimer of subunits reassociate with another subunit/dimer from a different tetramer. If the rate of subunit dissociation is high, but that of reassociation still higher, the equilibrium constant will favor the tetramer, keeping dimers and monomers at vanishingly low concentrations. Clearly, the rapid exchange of subunits observed here introduces many new considerations and another level of complexity regarding the assembly and regulation of FBPase.

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