Creation of an Allosteric Phosphofructokinase Starting with a Nonallosteric Enzyme

THE CASE OF DICTYOSTELIUM DISCOIDEUM PHOSPHOFRUCTOKINASE*

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An allosteric phosphofructokinase (PFK) was created by sequence manipulation of the nonallosteric enzyme from the slime mold Dictyostelium discoideum (DdPFK). Most amino acid residues proposed as important for catalytic and allosteric sites are conserved in DdPFK except for a few of them, and their reversion did not modify its kinetic behavior. However, deletions at the unique C-terminal extension of this PFK produced a markedly allosteric enzyme. Thus, a mutant lacking the last 26 C-terminal residues exhibited hysteresis in the time course, intense cooperativity ($n_H = 3.8$), and a 200-fold decrease in the apparent affinity for fructose 6-phosphate ($K_{cat} = 4500 \mu M$), strong activation by fructose 2,6-bisphosphate ($K_{act} = 0.1 \mu M$) and fructose 1,6-bisphosphate ($K_{act} = 40 \mu M$), dependence on enzyme concentration, proton inhibition, and subunit association-dissociation in response to fructose 6-phosphate versus the nonhysteretic and hyperbolic wild-type enzyme ($n_H = 1.0$; $K_m = 22 \mu M$) that remained as a stable tetramer. Systematic deletions and point mutations at the C-tail region of DdPFK identified the last C-terminal residue, Leu$^{343}$, as critical to produce a nonallosteric enzyme. All allosteric mutants were practically insensitive to MgATP inhibition, suggesting that this effect does not involve the same allosteric transition as that responsible for fructose 6-phosphate cooperativity and fructose bisphosphate activation.

Phosphofructokinase (PFK, EC 2.7.1.11) catalyzes the first irreversible reaction specific for glycolysis, the phosphorylation of fructose 6-phosphate (Fru-6-P) by MgATP, to produce fructose 1,6-bisphosphate (Fru-1,6-P$_2$) and MgADP. Extensive studies established PFK as an example of a complex allosteric enzyme whose activity is thought fundamental for the control of energy production and is tightly regulated by a variety of allosteric mechanisms (reviewed in Refs. 1–3). Thus, it shows a very high degree of positive cooperativity (sigmoidal kinetics) as well as a high $K_{cat}$ for Fru-6-P and is sensitive to a number of effectors. Fructose bisphosphates are the most potent regulators (reviewed in Ref. 4), although some isoforms, such as the mammalian C type (5) or that from yeast (6), are only sensitive to Fru-2,6-P$_2$ (active in the micromolar range). ATP binding to its inhibitory site is regarded as central for the function of PFK, since it influences the action of other ligands (7–9). Hysteresis in the time courses of PFK reaction is also characteristic of several eukaryotic isoforms (7, 10–12). The regulatory properties of this enzyme are pH and concentration dependent, which operate by altering its oligomeric structure. A tetramer was demonstrated to be the smallest active form of rabbit muscle PFK (13), which upon protonation (14) reversibly isomerizes to an inactive form and then slowly dissociates to dimers and monomers. Increasing concentrations of enzyme promote self-association that can lead to aggregates larger than tetramer, as reported for muscle (15) and liver (16) PFK. This phenomenon involves pronounced changes in the kinetic properties (9, 11, 15, 16) and can be important in accounting for its function in vivo (17).

The allosteric nature of PFK has been verified in the simple prokaryotic enzyme by three-dimensional structure and specific mutation analyses (Refs. 18–21, and references therein). However, our knowledge of the structural changes that support the allosteric behavior of eukaryotic PFK is considerably lower. This is a more complex form of enzyme in both structure and regulation (1, 2, 22) and no crystal structure for it has been provided yet. Eukaryotic PFKs are about twice the size of the bacterial enzyme as a result of duplication, fusion, and mutation of an ancestral prokaryotic gene, thus leading to the formation of new allosteric sites (22). Therefore, structure/function data from the prokaryotic enzyme are difficult to extrapolate to eukaryotic isoforms, although several site-directed mutagenesis studies to identify amino acid residues implicated in ligand-binding sites have been carried out recently with the latter PFK types (23–26). We have used the nonallosteric enzyme from the slime mold Dictyostelium discoideum (DdPFK) as an alternative way to investigate the molecular bases of control mechanisms of PFK from eukaryotic cells. Despite having a marked degree of similarity to other isoforms (27), DdPFK is unusual among eukaryotic PFKs in that it displays hyperbolic kinetics, exhibiting a high affinity for Fru-6-P and no sensitivity to any of the characteristic effectors of other isoforms (28). DdPFK is also devoid of a concentration-dependent activity, being a stable tetramer (28). Therefore, this enzyme is particularly suitable to undertake at least partial restoration of an allosteric behavior by mutation. In addition to leading to the structural reasons for its lack of specific control properties, this approach could provide valuable information into the molecular mechanisms underlying Fru-6-P...
cooperativity and its relation with the action of allosteric effectors. We have found that deletions at the C-tail region of DdPFK convert it into an allosteric enzyme, displaying intense Fru-6-P cooperativity, strong activation by fructose bisphosphates, and subunit association-dissociation, among other properties characteristic of regulatory PFKs, although remaining practically insensitive to ATP inhibition. Further analyses indicated that deletion, or substitution, of the final C-terminal residue is enough to generate an allosteric behavior. These findings provide new insight into the allosteric control of PFK.

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents and enzymes used in genetic assays and protein purification were obtained from Roche Molecular Diagnostics, Amersham Bioscience, Inc., or Sigma. The Sculptor™ in vitro Mutagenesis System for site-directed mutagenesis was purchased from Amersham Bioscience, Inc. Oligonucleotides were from MedProbe.

Site-directed Mutagenesis and Genetic Manipulations—Point mutations were created according to the manufacturer’s protocol for the Sculptor™. In vitro Mutagenesis System, using the single-stranded DNA derived from the plasmid pMDdPFK. This plasmid contained the full-length cDNA of DdPFK and was constructed by cloning the 2.7-kb BamHI fragment from our previously obtained pE3 (27) into the M13mp19 vector. The mutagenic primers used are described in Table I. C-terminal deletion mutants were constructed by PCR employing mutagenic primers that generated the TAA stop codon at the desired position and an EcoRI site at the 3’ end of the mutated pfk gene. To obtain the Δ26 mutant, the 634-bp fragment of the 3’ end of DdPFK cDNA was isolated from the plasmid pE3 (27) by NheI-digestion, blunt-ending, and SpeI-digestion, and then cloned into pBluescript II SK– that had been XbaI-digested, blunt-ended, and SpeI-digested yielding pMUT1, which was used as a template. The PCR product was Nhel-EcoRI-digested and ligated with a HindIII-Nhel fragment from pE3 (containing the remained 2002 bp of the 5’ end of DdPFK cDNA) and HindIII-EcoRI-digested pBluescript II SK– to give pMUT2. The plasmid pMUT1 was used as a template to generate all other deletion mutants and their corresponding PCR products were cloned as Nhel-EcoRI fragments into pMUT2. All mutants were verified by DNA sequencing. For expression in yeast, mutant pfk genes were inserted as BamHI fragments downstream of the PFK2 promoter of Saccharomyces cerevisiae in the plasmid pJH171 (29).

Expression and Purification of Recombinant Enzymes—The plasmids containing either wild-type (29) or mutant PFK cDNA were expressed in a S. cerevisiae strain HD152-1D (5) carrying deletions in both yeast PFK genes. Transformation of yeast cells, media, and carbon sources were as described previously (29). Yeast transformants were grown in 1 liter of rich medium containing glucose to early stationary phase and recombinant enzymes were purified by 10% (w/v) polyethylene glycol (PEG) fractionation and chromatography on DE52 and blue Sepharose CL-4B as described previously (29), except that PFK activity was eluted from the latter column with a 100-ml linear gradient of 0–1.5 M KCl in equilibration buffer. All final preparations of recombinant enzymes were judged to be homogeneous by SDS-PAGE analysis on 10% gels and Coomassie Blue staining (28) (Fig. 1).

Enzyme Assay—PFK activity was measured in an assay mixture that unless otherwise indicated contained 50 mM Hepes, 100 mM KCl, 5 mM MgCl₂, pH 7.2, 0.15 mM NADH, 1 mM MgATP, 1.2 units of aldolase, 10 units of triose-phosphate isomerase, 1 unit of glycerol-3-P dehydrogenase, and 5–10 μl of the purified enzyme in a total volume of 1 ml. After 5 min, the reaction was started by adding 1 ml Fru-6-P and was followed by measuring the absorbance change at 540 nm at 23 °C. When PFK activity was assayed in permeabilized cells, Glu-6-P was added to

**TABLE I**

| Mutation | Synthetic oligodeoxynucleotides (5’ to 3’) |
|----------|----------------------------------|
| S288R    | TCTTCCTCTCTCATTCACCC |
| A568S    | CTTGGTTTGAATGGTACC |
| T833A    | TATTTATAGCGAATATTGTGATAC |
| L834A    | TATTTATGCGAATATTGTGATAC |
| Δ26      | GGgaatteTTAATGACCTACCTTCGA |
| Δ36      | CTCgaattcTTAATGATCTATCTTGTCG |
| Δ34      | GGGgaatteTTACGCTGAAAGTTTTGAG |
| Δ2        | GGgaatteTTACGCTGAAAGTTTTGAG |
| Δ1        | GGgaatteTTACGCTGAAAGTTTTGAG |

The introduced mutations are in bold face. EcoRI restriction site is in lowercase. Stop codons are underlined.

**TABLE II**

| Enzyme | $K_{cat}$ | $V_{max}$ or $S_{0.5}$ | $n_{H}$ | $K_m$ | $n_{H}$ | $K_m$ | Inhibition by 10 mM MgATP | $K_{cat}^{d}$ | $V_{max}^{d}$ |
|--------|----------|-------------------|--------|-------|--------|-------|-----------------------|-------------|-------------|
|        | s⁻¹      | μM                | (Fru-6-P) | (Fru-6-P) | (MgATP) | (MgATP) | No inhibition | No activation | No activation |
| Wild-type | 236      | 22                | 1.1  | 17²   | 1.1  | 10² | No inhibition | No activation | No activation |
| S288R  | 182      | 25                | 1.0  | 22²   | 1.0  | 10² | No inhibition | No activation | No activation |
| A568S  | 187      | 25                | 1.1  | 17²   | 1.1  | 10² | No inhibition | No activation | No activation |
| Δ26    | 84       | 4,500             | 3.8  | 50²   | 1.0  | 59   | 0.1  | 40 |
| Δ34    | 90       | 2,500             | 2.8  | 70²   | 1.0  | 0.12 | 110 |
| Δ36    | 63       | 2,000             | 2.0  | 100²  | 1.0  | 0.2  | 200 |
| Δ4     | 53       | 5,000             | 3.8  | 40²   | 1.0  | 0.05 | 140 |
| Δ1     | 33       | 1,000             | 3.6  | 19²   | 1.0  | 0.11 | 150 |
| Δ2     | 52       | 2,500             | 3.6  | 19²   | 1.0  | 0.04 | 30 |
| Δ1     | 57       | 550               | 1.6  | 22²   | 1.0  | 0.02 | 12 |
| T833A  | 270      | 19                | 1.1  | 18²   | 1.0  | 0.05 | 50 |
| L834A  | 70       | 19                | 1.1  | 70²   | 1.1  | 0.05 | 10 |

$K_{cat}$ and $S_{0.5}$, substrate concentration at half-maximal velocity. $K_{cat}$ (Fru-6-P) refers to values obtained when $n_{H}$ (Fru-6-P) was about 1. $K_m$ or $S_{0.5}$ (Fru-6-P) values were determined at 3 mM MgATP.

$K_{cat}$ (Fru-6-P) values were obtained at 1 mM Fru-6-P.

$V_{max}$ (MgATP) values were obtained at 1 mM MgATP.

$K_{cat}^{d}$, effecter concentration at half-maximal activation. These values were obtained at 1 mM Fru-6-P concentrations equal to the $K_m$ or $S_{0.5}$ (Fru-6-P) values and 3 mM MgATP.

$K_{cat}^{d}$ (MgATP) values were obtained at 1 mM Fru-6-P.

$K_{cat}^{d}$ (MgATP) values were obtained at 1 mM Fru-6-P.
Fru-6-P in a proportion 3:1. When the effect of Fru-1,6-P2 was examined, pyruvate kinase (1 unit) and lactate dehydrogenase (1 unit) were used as coupling enzymes in the presence of 0.2 mM P-enolpyruvate. Auxiliary enzymes were desalted by centrifugation and dialysis against 10 mM Hepes, pH 7.0, 20% (v/v) glycerol. One unit of activity is defined as the amount that catalyzes the conversion of 1 mmol/min of substrate at 25°C.

**PFKL Size-exclusion Chromatography—**PFKL samples dialyzed against 50 mM NaH2PO4, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, pH 6.8, were applied to a Amersham Bioscience, Inc. Superose 6 HR 10/30 column equilibrated and eluted with the same buffer at a flow rate of 0.3 ml/min. When indicated, the elution buffer contained 6 mM Fru-6-P.

Other Methods—Protein was determined by the method of Bradford (30). Permeabilization of yeast cells was carried out with a toluene/Ethanol/Triton X-100 mixture as described in Ref. 31. Mass spectrometry of recombinant enzymes was performed on a Bruker (Bremen, Germany) Reflex II matrix-assisted laser desorption ionization-time of flight mass spectrometer. ELISA analysis was performed in antibody capture mode (32) using 1/2,000 dilution of polyclonal rabbit antibody against DdPFK (28) and assaying peroxidase (32); samples containing no antigen were used as a blank. Prediction of secondary structure was obtained from the PHD server (33).

**RESULTS**

A Deletion of the C-tail Region of the Nonallosteric DdPFK Converts It into an Allosteric Enzyme—DdPFK has been reported to share about a 40% identity with other isozymes (27). Most amino acid residues involved in putative binding sites of substrates and allosteric effectors are conserved in this enzyme, except for a few changes located at the sites assigned to ADP/AMP, ATP, and Fru-6-P binding sites (32). The initial approach to generate allosteric properties in DdPFK was to reverse the single changes by site-directed mutagenesis. The corresponding cDNAs were expressed in a PFK-deficient strain of *S. cerevisiae* (5) and enzyme mutants were purified (Fig. 1) and characterized. However, neither S288R and A568S mutants (Table II) nor the V775R mutant (to be reported elsewhere) exhibited kinetic properties significantly different from those of the nonallosteric wild-type enzyme, nor they were sensitive to other effectors such as AMP/ADP, citrate, P0, or NH4+.

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Sequence alignments of amino acid residues of selected regions of PFKs from various organisms showing the changes in ligand binding sites of the *D. discoideum* enzyme (asterisks). Sequences are from *D. discoideum* (Dd), mouse liver (Ml), rabbit muscle (Rm), ascites tumor cells (C-type) (At), *S. cerevisiae* (Sc), *Bacillus stearothermophilus* (B), and *Escherichia coli* (Ec). The bottom alignment comprises the C-tail region of listed PFKs. Amino acids identical to DdPFK are shaded. Black triangles indicate putative residues assigned to the binding of several allosteric effectors (22, 53). A nonapeptide located in the C-terminal end of rabbit muscle PFK (RmPFK), which was identified as the ATP inhibition site (34), is boxed. Arrows indicate deletions introduced in the DdPFK sequence.

The control properties of DdPFK could be related to: (i) other unidentified residues important for the binding of specific ligands; (ii) the changes observed at the putative ATP inhibitory site, since ATP inhibition was proposed as basic for PFK regulatory characteristics (9); or (iii) that some of the structural motifs unique to this isozyme prevent allosteric transitions in some way. Regarding the latter possibility, the highest variability among PFK sequences is exhibited at the C-tail region, from about position 770 of DdPFK (Fig. 2). Additionally, this enzyme contains a C-terminal extension of 20–40 amino acids with respect to other eukaryotic PFKs. A deletion of the 26 C-terminal residues (Δ26) was made in DdPFK to test the role of this extension and the mutant enzyme was obtained as above. Surprisingly, this mutant exhibited dramatic changes in its kinetic behavior versus the wild-type enzyme. Whereas the initial rate of product formation of wild-type DdPFK was linear (28), a pronounced lag phase before attaining the steady state, i.e. hysteresis (35), was observed with the Δ26 mutant when the assay was initiated with Fru-6-P (Fig. 3). The duration of the lag time (z) was in the order of minutes and decreased with increasing Fru-6-P concentration, which at 2.5 mM augmented +2-fold. This hysteretic behavior, typical of allosteric PFKs (7, 10–12), was not observed when the reaction was initiated with ATP and was not modified by increasing the concentration of coupling enzymes.

These data prompted a detailed characterization of the regulatory and physical properties of the deleted enzyme. As shown in Fig. 4, the Δ26 mutant exhibited an intense positive cooperativity for Fru-6-P with a Hill coefficient of 3.8 and a 200-fold decrease in the apparent affinity for this substrate with respect to the hyperbolic (nH = 1.0) wild-type enzyme (S0.5 and Km values of 4500 and 22 μM, respectively). As seen in Table II, Δ26 showed a 3-fold decrease in the Km value, which was not large, indicating that catalysis was not grossly affected by the deletion. In contrast to the nonallosteric wild-type enzyme, this mutant was strongly activated by 1 μM Fru-6-P, which shifted to the left the Fru-6-P saturation curve (Fig. 4), abolishing the cooperativity (nH = 1.05) and reducing the S0.5 value to 0.5 mM without significant effect on the Vmax. A Kmax value of 0.1 μM was obtained for Fru-2,6-P2 and a similar activation effect was observed with Fru-1,6-P2, but with a higher Kact value of 40 μM (Table II). The Δ26 mutant...
also exhibited a concentration-dependent activity as shown by including an aggregating agent, PEG (36), in the assay, this elicited an effect on the Fru-6-P saturation curve similar to that exerted by the addition of 1 \( \mu \text{M} \) Fru-2,6-P\(_2\). This observation was confirmed by studying the truncated enzyme in permeabilized cells (the mutant producing yeast), i.e. in situ, where the enzyme is concentrated and the \( S_{0.5} \) and \( n_H \) values decreased to 0.25 mM and 1.5, respectively, from 11 mM and 3.0, respectively, in a dialyzed cell-free extract assayed in dilute solution (data not shown). A concentration-dependent behavior is absent in the wild-type enzyme (28).

The binding of Fru-6-P to the \( \Delta 26 \) mutant, as analyzed by protection against thermal inactivation of the enzyme, was found to be cooperative with an \( n_H \) value of 3.7 and an apparent dissociation constant of 4.5 mM, whereas that of the wild-type enzyme was hyperbolic (Fig. 5). Thus, the kinetic curve of the mutant did practically not deviate from the binding curve, meaning that the enzymic cooperativity of \( \Delta 26 \) was due solely to equilibrium binding of Fru-6-P and not to change in the catalytic rate constants of the various enzyme-substrate complexes (37).

The kinetic properties of the \( \Delta 26 \) mutant with respect to MgATP were not significantly different from those of the wild-type enzyme (Table II), except that it was moderately inhibited by this compound (59% inhibition at 10 mM MgATP). Among other PFK effectors, neither ADP, AMP, citrate, \( P_i \), nor \( P\)-enolpyruvate modified the activity of this mutant assayed at 3 mM Fru-6-P and 5 mM MgATP, and only a 66% activation was elicited by \( \text{NH}_4^+ \), each of these compounds being used at a concentration of 2 mM (data not shown).

Proton inhibition of animal PFK is well known to work by decreasing Fru-6-P affinity (7), which can be concomitant with an increase in cooperativity (38), and by increasing the affinity for binding of MgATP to its inhibitory site (39). Fig. 6 shows the inhibitory effect of pH on the Fru-6-P saturation curve of two different C-terminal deletions. An increase in the cooperativity of the \( \Delta 26 \) mutant was apparent, although not intense, when lowering the pH value from 8.0 to 7.2 or 6.4 (Fig. 6A). This effect was more noticeable (Fig. 6B) in a \( \Delta 8 \) mutant (see below), which kinetic behavior was similar to that of \( \Delta 26 \) but was insensitive to ATP inhibition (Table II). Apparent affinity of these mutants for Fru-6-P was less significantly affected. The kinetics of the wild-type enzyme was reported (28) to remain hyperbolic even at pH 5.5. It is noteworthy that in addition to high Fru-6-P concentration, all the above conditions that de-
creased Fru-6-P cooperativity of the Δ26 mutant, also decreased hysteresis in the time courses of the PFK reaction (data not shown).

The oligomeric state of the Δ26 mutant was analyzed by FPLC size-exclusion chromatography, to test its relation with enzyme function. As shown in Fig. 7, the truncated DdPFK eluted basically as inactive dimers and monomers that slowly reactivated upon incubation with 5 mM Fru-6-P. In addition, the Δ26 mutant stabilized into an active tetramer when chromatographed in the presence of this ligand. In contrast, the wild-type enzyme eluted as an active tetrameric form independently of the presence of Fru-6-P. Increasing the concentration of KCl from 0.1 to 0.5 M in the equilibration and elution buffers, which favors protein-protein interactions, also shifted the association-dissociation equilibrium of the mutant enzyme to the active tetramer, which then contributed 61% to the eluted protein (data not shown). This behavior is consistent with the dilution-mediated inactivation/dissociation of muscle (40) and liver (41) PFK prevented by Fru-6-P.

Identification of the C-terminal Motifs Involved in the Lack of Allosteric Properties of DdPFK—C-terminal deletions of longer (Δ36) and shorter (Δ14) extension than Δ26 were first carried out for a structure/function characterization of the C-tail region of DdPFK. Analysis of their properties led us to construct progressively shorter deletions, in combination with single-point mutations, to identify the structural motifs responsible for the role of this region in preventing allosteric transitions.

Table II shows that deletion mutants Δ36, Δ14, Δ8, Δ4, and, interestingly, even Δ2, exhibited characteristics that were very close to those of the Δ26 mutant, i.e. low apparent affinity (S0.5 values from 5 to 1 mM) and cooperative kinetics for Fru-6-P (nH values from 3.8 to 2.0), strong activation by Fru-2,6-P2 (Kact values from 0.04 to 0.2 mM) and Fru-1,6-P2 (Kact values from 30 to 200 mM), no significant change in MgATP kinetics with respect to the wild-type enzyme, and Kcat values within the same range. None of these deletion mutants showed MgATP inhibition. These data suggested an important function for either, or both, of the last two C-terminal amino acids of DdPFK in accounting for its peculiar behavior. Deletion of the last residue, Leu834 (Δ1 mutant), was enough to generate an allosteric PFK sensitive to a very potent stimulation by Fru-2,6-P2/Fru-1,6-P2 (Kact values of 0.02 and 12 mM, respectively), although with lower degree of cooperativity (nH = 1.6) and some higher apparent affinity for Fru-6-P (S0.5 = 0.55 mM) than...
the Δ26 mutant (but still 1 order of magnitude lower than that of the hyperbolic wild-type enzyme). Replacement of this residue by an alanine (L834A mutant) virtually reproduced the kinetic behavior of the Δ1 mutant, whereas a similar mutation of the one before last residue (T833A mutant) did not introduce any change in the kinetic properties of the wild-type enzyme. This confirms the critical role of Leu<sup>834</sup> among the last two residues of the C-tail of DdPFK.

**DISCUSSION**

In this study, we have introduced specific mutations in the sequence of the nonregulatory PFK from *D. discoideum* to generate an allosteric behavior. A deletion of the 26 C-terminal residues resulted in drastic changes in the kinetic and physical properties of the enzyme allowing it with regulatory capabilities, that were qualitatively and quantitatively similar to those characteristic of allosteric PFKs (1–3, 5, 7, 10).

The hysteretic response of the Δ26 mutant to Fru-6-P correlated well with cooperativity. Thus suggesting that both phenomena represent the same process, as shown for regulated PFKs (12, 42). The question arises as to how the marked cooperativity in Fru-6-P binding to the mutant enzyme is generated. The truncated DdPFK is subject to subunit dissociation/reassociation in dependence of this ligand (Fig. 7), or ionic strength. However, reactivation to a tetramer by Fru-6-P took much longer (at least 60 min for partial reactivation) than the time required to measure initial velocity. The purified mutant enzyme must be predominantly in this form, since it eluted from the last chromatography column at 0.7 M KCl and over 60% of the protein behaved as a tetramer on FPLC size-exclusion chromatography in 0.5 M KCl. These data indicate that the minimal active form of the Δ26 mutant in the kinetic assay is the tetramer, and that subunit reassociation is not directly involved in hysteresis and cooperativity. The great decrease in cooperativity observed with the aggregating agent PEG, or in situ, paralleled the behavior reported for mammalian PFKs under similar conditions (9, 17, 43) or at increasing concentrations of enzyme that promoted aggregation beyond the active tetramer (15, 16, 36), thus suggesting that subunit interactions in the mutant DdPFK are large enough to elicit polymerization of the tetramer. Ligand-linked changes in the self-association equilibria of the oligomeric enzyme could also originate cooperativity (37). Nevertheless, aggregation of the truncated enzyme is not expected during the regular kinetic assay, since enzyme concentration was 1–2 μg/ml and no species larger than the tetramer were detected by FPLC gel filtration performed at a protein concentration of 300 μg/ml in the presence of Fru-6-P (Fig. 7). Therefore, our results indicate that cooperativity, and hysteresis occur within the tetramer and that it is mediated by the isomerization of a less active conformation (T) to a more active one (R) as Fru-6-P shifts the equilibrium toward the favoring binding conformation (R). The fact that wild-type DdPFK is locked in a stable tetrameric R-like conformation supports that subunit interactions underlying the R/T transition eventually lead to reversible subunit dissociation. This interpretation coincides with the model of Frieden and co-workers (14, 38, 40) for the regulation of muscle PFK. Accordingly, proton inhibition of the deleted enzyme fits the proposal of these authors stating that protonation of certain ionizable groups promotes inactivation by isomerization of the tetramer. Nevertheless, the pH effect on deleted PFKs (Fig. 6) was less intense than those exhibited by animal PFKs (7, 12, 38), reflecting mostly a change on the protonation state of catalytic groups at the active site. Since MgATP inhibition was scarce or absent in DdPFK mutants, this suggests that proton enhanced binding of MgATP at its inhibitory site (39) contributes in a major extent to the decrease in Fru-6-P affinity and/or cooperativity upon protonation of animal PFKs (7, 12, 38).

The above description of Fru-6-P cooperativity of deletion mutants is consistent with the two-state concerted model of allosteric regulation (44). The interdependence of this phenomenon and MgATP inhibition led several authors (7, 38, 45) to interpret cooperativity in terms of the heterotropic interactions (44) between binding of Fru-6-P and MgATP at the active site and inhibitory site, respectively. This interpretation was also supported by chemical (46–48) and proteolytic (34) modifications of eukaryotic PFKs that abolished or reduced both properties to a similar extent. However, our results with truncated DdPFKs (Table II) show that it is possible to have a highly cooperative enzyme completely insensitive to inhibition by MgATP. Although this observation was made by endowing with cooperativity a previously nonallosteric PFK, rather than by eliminating MgATP inhibition from a normal allosteric form, it suggests that these two phenomena can be decoupled and therefore may not be related to the same allosteric transition as...
required in the concerted model of Monod et al. (44). In fact, a similar suggestion was previously made by Rao et al. (49) with the allosterically regulated PFK from Ascaris suum. Nevertheless, this does not imply that both properties do not interact to provide a finer modulation of PFK activity. The mechanistic separation between cooperativity and MgATP inhibition may explain several previous observations that did not follow heterotropic cooperativity. For instance, (i) animal (17) or yeast (50) PFK exhibits normal cooperativity when ITP is used as a phosphoryl donor, despite that this compound is not a PFK inhibitor (8, 51), and (ii) cooperativity of yeast PFK does not substantially change at different degrees of MgATP inhibition (31).

Although DdPFK is not activated by Fru-2,6-P₂, it efficiently binds this compound (Kᵟ of allosteric regulation (44). Although the Fru-2,6-P₂/Fru-1,6-P₂ transition that accounts for cooperativity, analogously to the concerted model of allosteric regulation (44). Although the Fru-2,6-P₂/Fru-1,6-P₂ activation of PFK is clearly affected by MgATP inhibition (1, 4), our data indicate that the actions of fructose bisphosphates and MgATP are also not coupled, i.e. that they are not brought about by a unique R/T transition as assumed by the model of Monod et al. (44). Reversion of the change R775V at the putative ATP inhibition site did not re-establish this property in DdPFK. Therefore, it is tempting to relate its lack to the absence of a C termini nonapeptide sequence (Fig. 2) identified as crucial for ATP inhibition (34). This in turn could also be related to the insensitivity of DdPFK to inhibitors that act synergistically with MgATP, like citrate or P-enolpyruvate (8). Nevertheless, our results indicate that the proposal of ATP inhibition as the basic mechanism for the operation of PFK regulatory characteristics (9) was an oversimplification, since at least some of them, as the effect of enzyme concentration, fructose bisphosphates activation and, in part, proton inhibition, operate on Fru-6-P cooperativity in a way primarily independent of inhibition by MgATP.

The findings presented here show that the unique C-terminal extension of DdPFK (Fig. 2) is responsible for its lack of allosteric transitions. Systematic sequence manipulation of this region (a predicted loop) suggests that its function is to serve as an arm to position the last leucine residue, so that the latter hydrophobically interacts with some unknown group to maintain the protein as a stable tetramer. Thus, evolution to generate the hyperbolic behavior of DdPFK appears to have operated not by additional point mutations but by the acquisi-

tion of an extra C-terminal sequence, that somehow locks the enzyme in an R-like conformation preventing the intersubunit communication responsible for cooperativity (44, 52) and reversible subunit dissociation. Identification of the involved amino acid interactions and elucidation of the structural changes brought about by the C-terminal tail of DdPFK awaits determination of the three-dimensional structure of both the wild-type enzyme and at least some of the allosteric truncated mutants.

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Creation of an Allosteric Phosphofructokinase Starting with a Nonallosteric Enzyme: THE CASE OF DICTYOSTELIUM DISCOIDEUMPHOSPHOFRUCTOKINASE
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