Distinct functional roles of the two terminal halves of eukaryotic phosphofructokinase

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Eukaryotic PFK (phosphofructokinase), a key regulatory enzyme in glycolysis, has homologous N- and C-terminal domains thought to result from duplication, fusion and divergence of an ancestral prokaryotic gene. It has been suggested that both the active site and the Fru-2,6-P₂ (fructose 2,6-bisphosphate) allosteric site are formed by opposing N- and C-termini of subunits orientated antiparallel in a dimer. In contrast, we show in the present study that in fact the N-terminal halves form the active site, since expression of the N-terminal half of the enzymes from Dictyostelium discoideum and human muscle in PFK-deficient yeast restored growth on glucose. However, the N-terminus alone was not stable in vitro. The C-terminus is not catalytic, but is needed for stability of the enzyme, as is the connecting peptide that normally joins the two domains (here included in the N-terminus). Co-expression of homologous, but not heterologous, N- and C-termini yielded stable fully active enzymes in vitro with sizes and kinetic properties similar to those of the wild-type tetrameric enzymes. This indicates that the separately translated domains can fold sufficiently well to bind to each other, that such binding of complementary domains is stable and that the alignment is sufficiently accurate and tight as to preserve metabolite binding sites and allosteric interactions.

Key words: enzyme engineering, enzyme regulation, ethanol fermentation, glycolysis, phosphofructokinase, structure–function relationship.

INTRODUCTION

PFK (6-phosphofructo-1-kinase; EC 2.7.1.11) is a key regulatory enzyme in the glycolytic pathway, converting Fru-6-P (fructose 6-phosphate) into Fru-1,6-P₂ (fructose 1,6-bisphosphate). The mammalian enzyme is controlled by a variety of allosteric effectors, including the inhibitors ATP and citrate, and the activators AMP and ADP [1]. The most potent activator is Fru-2,6-P₂ (fructose 2,6-bisphosphate), which is especially important in the reciprocal control of glycolysis and gluconeogenesis in the liver, as Fru-2,6-P₂ also inhibits the opposing gluconeogenic enzyme fructose-1,6-bisphosphatase [2]. PFK from eukaryotic cells is composed of two highly homologous N- and C-terminal domains, which are thought to have resulted from duplication, fusion and divergence of an ancestral prokaryotic gene. This evolutionary process would lead to the formation of new regulatory binding sites. Thus the site for the allosteric activator Fru-2,6-P₂ is suggested to derive from the Fru-6-P catalytic site in the duplicated bacterial repeat, the two sites being located in the interface between the subunits of a dimer [3].

However, an original proposal was that, in the eukaryotic enzyme, the chains were orientated in an antiparallel manner, such that the active site was in the interface between an N-terminal half and an adjacent C-terminal half, using amino acid residues from both, and similarly for the Fru-2,6-P₂ binding [3–5]. On the other hand, our previous work with site-directed mutagenesis showed that key residues for the active site were in the N-terminus, whereas key residues for the Fru-2,6-P₂ site were in the C-terminus, suggesting a N-terminal/N-terminal–C-terminal/C-terminal interaction of adjacent chains [6]. This model is consistent with the recent reports of the electron microscopy structure of human muscle PFK [7] and the crystal structures of the enzymes from the yeasts Pichia pastoris [8] and Saccharomyces cerevisiae [9]. In the present paper we describe conclusive evidence that the N-terminal half, by itself, is capable of forming a functional PFK active site. We expressed several truncated forms of the enzymes from DdPFK (Dictyostelium discoideum PFK) (Figure 1) and human muscle (HmPFK) in a PFK-deficient strain of S. cerevisiae. We chose the non-allosteric DdPFK for most of the present study to take advantage of its much greater stability and ease of purification [11]. The C-terminus is not catalytic, but it is needed for stability of the enzyme, as is the connecting peptide that joins the two domains.

EXPERIMENTAL

Materials

All chemical reagents and enzymes used in genetic assays and protein purification were obtained from Roche, GE Healthcare or Sigma. Auxiliary enzymes and biochemicals for the PFK assay were from Sigma. Oligonucleotides were synthesized by...

Abbreviations used: C-term, transformants expressing the sequence for the C-terminal half of phosphofructokinase; DdPFK, Dictyostelium discoideum phosphofructokinase; Fru-2,6-P₂, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; HmPFK-M, human muscle phosphofructokinase; N-term, transformants expressing the sequence of the N-terminal half of phosphofructokinase; PFK, phosphofructokinase; ChiDd–PFK, chimaeric PFK composed of the N-terminal half of DdPFK and the C-terminal half of HmPFK-M; ChiHm–PFK, chimaeric PFK composed of the N-terminal half of HmPFK-M and the C-terminal half of DdPFK; pG-term, C-term containing the peptide N-(370–436) at the N-terminal end; YNB, yeast nitrogen base.

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Isogen or Sigma. Antibodies against the M subunit of rat muscle PFK were donated by Dr G.A. Dunaway (Southern Illinois University, Carbondale, IL, U.S.A.). Horseradish-peroxidase-conjugated goat anti- (rabbit immunoglobulin G) was from Nordic Bioscience. Other reagents were obtained from commercial sources and were of the best grade available.

Strains, media and growth conditions

The *Escherichia coli* strain DH5α [SupE44 ΔlacU169 (φ80 lacZΔM15) hisD17 recA1 endA1 gyrA96 thi-1 relA1] was used for general cloning procedures and amplification of DNA. *E. coli* was grown at 37°C in either liquid or solid LB (Luria–Bertani) media [12] with 50 μg/ml ampicillin when necessary. The *S. cerevisiae* strains used were: VW1a (MATα ura3-52 leu2-3, 112 his3Δ1 trpl-1-289 MAL2-8 SUC2 GAL) and the pk1 pk2 mutant HD152-1D (MATα pk1::HIS3 pk2::HIS3 ura3-52, his3-Δ1 leu2-3,112 trpl-1-289 MAL2-8 SUC2 GAL, [13]). Yeast strains were grown at 30°C in YPD medium [1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose] or in minimal medium containing 0.67% (w/v) YNB (yeast nitrogen base; Difco), 2% (w/v) of the carbon sources indicated in each case, and substances needed for the auxotrophic strain used at concentrations given previously [14]. Liquid cultures were shaken at 200 rev./min in a New Brunswick orbital shaker. Solid media were identical, but with 2% (w/v) agar added. Transformation of *S. cerevisiae* was performed as described in Klebe et al. [15], with the modifications introduced by Dohmen et al. [16]. Yeast generation times were measured by following the D600.

Molecular biology techniques and plasmid construction

Recombinant DNA manipulations were carried out as described previously [12]. N-terminal and C-terminal-truncated genes of DdPFK, and HmPFK-M were constructed by PCR amplification [17], using either the pJH71 derivative pBEΔ0 [11] carrying the cDNA for DdPFK or the plasmid pJH71PFK [18] containing HmPFK-M cDNA, as the template, and the mutagenic oligonucleotides indicated in Supplementary Table S1 (at http://www.BiochemJ.org/bj/445/bj4450213add.htm) in combination with the appropriate primer at either the 5′S1 (at http://www.BiochemJ.org/bj/445/bj4450213add.htm) in mutagenic oligonucleotides indicated in Supplementary Table containing HmPFK-M cDNA, as the template, and the cDNA for DdPFK or the plasmid pJJH71PFK [18] for general cloning procedures and amplification of DNA. *Escherichia coli* for general cloning procedures and amplification of DNA. *Escherichia coli* was grown at 37°C in either liquid or solid LB (Luria–Bertani) media [12] with 50 μg/ml ampicillin when necessary. The *S. cerevisiae* strains used were: VW1a (MATα ura3-52 leu2-3, 112 his3Δ1 trpl-1-289 MAL2-8 SUC2 GAL) and the pk1 pk2 mutant HD152-1D (MATα pk1::HIS3 pk2::HIS3 ura3-52, his3-Δ1 leu2-3,112 trpl-1-289 MAL2-8 SUC2 GAL, [13]). Yeast strains were grown at 30°C in YPD medium [1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose] or in minimal medium containing 0.67% (w/v) YNB (yeast nitrogen base; Difco), 2% (w/v) of the carbon sources indicated in each case, and substances needed for the auxotrophic strain used at concentrations given previously [14]. Liquid cultures were shaken at 200 rev./min in a New Brunswick orbital shaker. Solid media were identical, but with 2% (w/v) agar added. Transformation of *S. cerevisiae* was performed as described in Klebe et al. [15], with the modifications introduced by Dohmen et al. [16]. Yeast generation times were measured by following the D600.

Expression and purification of recombinant enzymes

N-term represents transformants expressing the sequence of the N-terminal half and C-term represents transformants expressing the sequence for the C-terminal half of PFK. For the production of N-term and C-term, the yeast strain HD152-1D transformed with the corresponding pJH71 and pGAD424 derivatives was grown to stationary phase in 2 litres of YNB medium with 2% glucose. For purification of the recombinant enzyme, first fractionation on 10% (w/v) poly(ethylene glycol) and chromatography on DE52 and Blue-Sepharose CL-6B was carried out as described previously [19], 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 [50 mM Hepes, 5 mM MgCl2, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF and 20% (w/v) glycerol, pH 6.8]. The dialysed Blue-Sepharose PFK fraction was then applied to a phosphocellulose column, this chromatographic step being performed as described in [20]. Fractions showing the highest PFK activity were pooled, concentrated 20-fold in dialysis tubing placed in powder poly(ethylene glycol) 6000, and subjected to FPLC size-exclusion chromatography on a SuperdexTM 200HR 10/30 column equilibrated with 50 mM sodium phosphate buffer, 150 mM NaCl and 10 mM Tris-Cl (pH 7.0). [21]. The recombinant PFK from yeasts expressing the N- and C-terminal regions of HmPFK-M was purified from yeast transformants grown to stationary phase in 2 litres of YNB medium with 2% glucose by chromatography on Blue-Sepharose CL-6B and DEAE-trisacryl performed as described previously [17]. Wild-type DdPFK and HmPFK-M were purified as described previously [11,17]. The protein concentration was determined by Bradford’s dye-binding method [22], using bovine γ-globulin as the standard.

PFK activity assay

Total PFK activity was measured as described previously [17]. Assays for kinetic studies were carried out at pH 7.0 in 50 mM Hepes, 100 mM KCl, 5 mM MgCl2, 0.15 mM NADH, 1.2 units of aldolase, 10 units of triosephosphate isomerase, 1 unit of glyceral-3-phosphate dehydrogenase, 2–5 μl of the purified enzyme, and the indicated concentrations of MgATP, effector and Fru-6-P in a final volume of 1 ml. In all cases, the reaction was started after a 5 min pre-incubation by the addition of Fru-6-P, and was followed by measuring the absorbance change at 340 nm at 25°C. When PFK activity was assayed during purification, glucose 6-phosphate was added to Fru-6-P at a proportion of 3:1 (mol/mol). Auxiliary enzymes were desalted as described previously [20]. One unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min under the above conditions. Kinetic data are the average of a minimum of three measurements. The standard error did not exceed 10%...
of the average value. Parameters were determined using Origin version 7.0 software programmed to fit data to either the Hill or the Michaelis–Menten equation.

Electrophoretic and immunological analysis

SDS/PAGE (10 % gels), Coomassie Blue staining and Western blot analysis were carried out as described previously [20]. Antiserum against DdPFK [20] or HmPFK-M, and the horseradish-peroxidase-conjugated secondary antibody were used at 1:3000 and 1:5000 dilutions respectively. Immunostaining was performed either as described by Martinez-Costa et al. [20] or with an ECL (enhanced chemiluminescence) detection technique [23]. ELISA analyses of FPLC size-exclusion chromatography fractions were performed as described previously [24], using a 1:10000 dilution of primary antibodies.

Glucose and ethanol measurements

To measure glucose consumption and ethanol production, yeast cells grown in YPD medium were collected during the exponential phase of growth, resuspended at 40 mg (wet weight)/ml in fresh medium containing 2 % glucose, and incubated with shaking at 30°C. Culture samples were taken at the indicated time, centrifuged at 3000 g for 1 min, and ethanol and glucose in the medium were measured by standard enzymatic techniques [25].

RESULTS

We found that the N-terminal half of DdPFK corrected the inability of the PFK-deficient recipient strain to grow on glucose, whereas the C-terminal half was ineffective (Figure 2). Western blot analysis confirmed the presence of truncated N- or C-termini in the respective transformants (Figure 3A). Yeast transformants expressing the N-terminus were indeed able to ferment glucose (Figure 4), thus demonstrating that this domain was sufficient to generate an active enzyme in intact cells. In fact, the N-terminal half was able to maintain nearly normal glucose metabolism in vivo. Thus yeast transformants expressing the N-terminus consumed glucose and produced ethanol at rates that were nearly 35 % of those of transformants expressing wild-type DdPFK (Figure 4, inset). The stoichiometric ethanol/glucose ratio (1.7) was similar in both cases and similar also to that shown by the isogenic untransformed strain VW1a which is wild-type for the yeast PFK genes. The glucose fermentation flux in the latter was approximately half of that of the HD152-1D strain expressing wild-type DdPFK, perhaps in part because of the non-allosteric hyperbolic nature of DdPFK [11,20] compared with the allosteric yeast PFK, which is thus subject to regulation in vivo [26]. The N-terminal half of DdPFK was, however, extremely unstable when assayed in a cell-free extract, rapidly becoming inactive (Supplementary Figure S1 at http://www.BiochemJ.org/bj/445/bj4450213add.htm). Size-exclusion chromatography showed that the N-terminus aggregated in cell extracts into (inactive) forms higher than octamers (equivalent to the native enzyme tetramer) (Figure 3B).

These findings are consistent with our hypothesis that the catalytic sites of eukaryotic PFK are restricted to the N-terminal half, which is capable of achieving a functional conformation in vivo reminiscent of the prokaryotic enzyme. Co-expression of the two terminal halves improved the growth of transformants, compared with expression of the N-terminus alone (Supplementary Figure S2 at http://www.BiochemJ.org/bj/445/bj4450213add.htm). Furthermore, co-expression of the two terminal halves generated a stable fully active enzyme that could be purified to homogeneity.
Table 1  Steady-state kinetic parameters of purified recombinant PFKs obtained by co-expressing the N-terminal and C-terminal regions from either DdPFK or HmPFK-M

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>DdPFK</th>
<th></th>
<th></th>
<th>HmPFK-M</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Kakt (s⁻¹)</td>
<td>236</td>
<td>304</td>
<td>357</td>
<td>356</td>
<td></td>
</tr>
<tr>
<td>S₀.5 (Fru-6-P) (μM)</td>
<td>0.022</td>
<td>0.025</td>
<td>2.0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>nH (Fru-6-P)†</td>
<td>1.1</td>
<td>1.1</td>
<td>3.0</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Kₐ (MgATP) (μM)‡</td>
<td>17</td>
<td>15</td>
<td>70</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>nH (MgATP)†</td>
<td>1.1</td>
<td>1.1</td>
<td>3.0</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Kᵢ (MgATP) (mM)‡</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>3.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Kact (Fru-2,6-P₂) (μM)§</td>
<td>No activation</td>
<td>No activation</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

* S₀.5 , and Kᵢ , substrate concentration at half-maximal velocity. S₀.5 (Fru-6-P) values were determined at 3 and 5 mM MgATP for DdPFK and HmPFK respectively, pH 7.2. Kᵢ (MgATP) values were obtained at Fru-6-P concentrations equal to the corresponding S₀.5 (Fru-6-P) values, pH 7.0. nH, Hill coefficient obtained by fitting the data to the Hill equation. † Kakt, MgATP concentration at half-maximal activation. These values were obtained at Fru-6-P concentrations equal to the S₀.5 (Fru-6-P) values, pH 7.0. § Kact, effector concentration at half-maximal activation. These values were obtained at Fru-6-P concentrations equal to the S₀.5 (Fru-6-P) values and 5 mM MgATP, pH 7.0. || Inhibitory MgATP concentrations were used to demonstrate the elevated S₀.5 and nH for Fru-6-6-P₂ and also Fru-2,6-P₂ activation of the mammalian enzyme.

Figure 5  Size-exclusion FPLC and SDS/PAGE of co-expressed N- and C-terminal halves of PFK

(A) Purified PFK from transformants expressing the two terminal halves of the D. discoideum enzyme were chromatographed on a Superdex 200HR 10/30 column. Fractions were assayed for enzyme activity (●) and ELISA (○). Arrows indicate the theoretical elution volumes of PFK forms corresponding to octamer, tetramer, dimer and monomer (composed by 8, 4, 2 and single N/C-terminal halves) and their calculated molecular mass values in kDa. (B) SDS/PAGE of purified co-expressed termini of DdPFK and PFK-M from human muscle (HmPFK-M). Samples containing 1 μg of purified enzymes were loaded per lane. Positions of molecular mass markers are shown on the right-hand side.

and was shown to adopt an octameric form of a molecular mass equivalent to that of the native enzyme (Figure 5A); the two terminal components being present in similar amounts as visualized by SDS/PAGE (Figure 5B). Therefore the oligomer was composed of four ‘N-terminal subunits’ and four ‘C-terminal subunits’, presumably organized into a conformation analogous to that of the tetrameric wild-type enzyme. The purified PFK activity obtained by co-expression of the two terminal halves of DdPFK exhibited kinetic properties similar to those of the wild-type enzyme (Table 1).

Similar results were obtained upon co-expression of the terminal halves of HmPFK-M, which also generated an active enzyme in vitro that furthermore exhibited binding at ATP and Fru-2,6-P₂ regulatory sites comparable with that of the intact enzyme (Figure 5B and Table 1), whereas expression of the N-terminus alone yielded an active enzyme in vivo, but not in vitro. The N-terminal half of HmPFK-M was even more unstable in cell-free extracts than that of DdPFK (Supplementary Figure S1). It is worth noting that the hardly detectable activity of the N-terminus of HmPFK-M was not modified by the addition of 10 μM Fru-2,6-P₂. This is consistent with the fructose bisphosphatase allosteric site being located in the C-terminal half of the enzyme [6]. Stability of the N-terminal half was not improved by addition of 10% poly(ethylene glycol), which increases the local concentration of the protein [27].

These results indicate that the C-terminal half of eukaryotic PFK, although devoid of a catalytic function, is essential for the enzyme to reach a stable tetrameric conformation. Interestingly, co-expression of the heterologous terminal domains (the DdPFK N-terminus with the HmPFK C-terminus, or the HmPFK N-terminus with the DdPFK C-terminus) did not lead to stable activity in vitro. Even chimaeric PFKs, involving exchange of the N- and C-terminal halves of DdPFK and HmPFK-M, did not produce stable activity in vitro. Of the two chimaeric constructs, only ChiDd–PFK was able to complement the glucose-negative phenotype of the yeast strain, producing an immunologically detectable protein of the expected size (Supplementary Figure S3 at http://www.BiochemJ.org/bj/445/bj4450213add.htm). Although this chimaeric enzyme was inactive in vitro, a significant proportion of it was able to organize into tetramers (Supplementary Figure S4 at http://www.BiochemJ.org/bj/445/bj4450213add.htm), like native PFKs. The other chimaeric construct ChiMDd–PFK apparently did not lead to sufficient active enzyme in vivo, indicating that a mismatched C-terminus can even destabilize the N-terminus.

The truncated N-terminal protein included the peptide that connects the two PFK domains in the native enzyme (residues 371–435). We therefore also examined the importance of this region. Expression of the N-terminus lacking the connecting peptide, N-(1–370), whether co-expressed with a C-terminus either lacking this peptide (C-term) or containing this peptide at its N-terminal end (pC-term), did not restore growth on glucose (Figure 6). Even partial deletions of the connecting peptide on the N-terminus [N-(1–396) and N-(1–420)] prevented the generation of functional active enzyme (Supplementary Figure S5 at http://www.BiochemJ.org/bj/445/bj4450213add.htm). These results indicate that the peptide which connects the two terminal domains of eukaryotic PFK is essential for the production of
a stable catalytic protein. Interestingly, C-term was no longer detected when co-expressed with N-(1–370).

**DISCUSSION**

A major conclusion of the present study is that the catalytic site of PFK is contained in the N-terminal half, with no necessary participation of C-terminal residues. This was conclusively shown by the restoration of growth on glucose in PFK-deficient yeast upon expression of the N-terminal half alone, whereas no growth was elicited by expression of the C-terminal half. This agrees with our previous studies with site-directed mutagenesis of key residues in the two halves [6]. Since the catalytic site is in the interface between subunits in a dimer, with residues contributed from both subunits, this indicates that subunits associate within a dimer by each terminal half binding to its equivalent one in the adjacent subunit. This conclusion is also consistent with the electron microscopy structure of human muscle PFK [7] and the crystal structures of the enzymes from the yeasts *P. pastoris* [8] and *S. cerevisiae* [9]. A similar assembly of the enzyme dimer can be inferred from the crystal structure of rabbit muscle PFK [9]. However, the N-terminal half by itself was extremely unstable when assayed in a cell-free extract, rapidly becoming inactive, which thus prevented further kinetic studies. Recently Šmerek et al. [28] reported that expression of a longer N-terminal fragment of HmPFK-M (residues 1–443, including the first 40 amino acids of the C-terminus) in a PFK-null *E. coli* strain allowed growth on glucose and produced a truncated enzyme that was also extremely unstable *in vitro*. In the present study we show that co-expression of the C-terminal half, together with the N-terminal half, yielded enzyme activity that was stable *in vitro*. This was true both for the non-allosteric DdPFK and for the allosteric mammalian HmPFK-M, thus indicating also that, despite its lack of most regulatory properties characteristic of eukaryotic PFK, the *D. discoideum* enzyme has a similar functional structure, with similar stabilizing interactions. This is consistent with the similar tetrameric organization for DdPFK, as suggested by electron microscopy analysis [7]. The underlying similarity is also supported by the interesting observation that deletion or mutation of the last C-terminal residue of DdPFK can convert it into an allosteric enzyme [11].

The fact that co-expression of the two terminal halves of PFK resulted in a fully active enzyme *in vitro*, with physical, kinetic and regulatory properties similar to those of the native enzyme (Figure 5 and Table 1), also shows that the interdomain interactions are strong enough to maintain the tetramer together, even after splitting of the connecting peptide, with preservation of the metabolite binding sites located at the corresponding interfaces. These results are consistent with the early observation by Emerk and Frieden [29] that trypsin treatment of muscle PFK gave two species of molecular masses 42,000 and 48,000 Da, without alteration of the regulatory characteristics of the enzyme. However, the situation in the present study is even more extreme, indicating that the separately translated domains can fold sufficiently well to bind to each other, that such binding of complementary domains is stable and that the alignment is sufficiently accurate and tight as to preserve allosteric interactions.

Interestingly, this stabilization effect was only seen with complementary enzyme halves; that is, heterologous expression of the DdPFK N-terminus with the HmPFK C-terminus, or the HmPFK N-terminus with the DdPFK C-terminus, did not produce a stable enzyme *in vitro*. Even linked chimaeric forms did not produce a stable enzyme *in vitro*, and one (the N-terminus of HmPFK-M linked to the C-terminus of DdPFK HmPFK-M) was not even active *in vivo*, indicating that the covalent attachment of a mismatched C-terminus could even destabilize an N-terminal half that was otherwise functional *in vivo*. In contrast, we previously reported studies of stable chimaeric forms of the mammalian isoforms PFK-M and PFK-C [30]. Thus the C-terminal domain appears to stabilize the whole enzyme to a substantial extent only when in combination with the N-terminal domain from either the same protein or from an evolutionarily close isoenzyme. This role of the C-terminal domain to stabilize the enzyme is, of course, in addition to its role in providing allosteric regulatory sites. Furthermore, the C-termini may provide the contact surfaces for the interaction between dimers within the tetramer, as proposed by the electron microscopy three-dimensional reconstruction of HmPFK-M [7] and shown in the crystal structure of the enzyme from *P. pastoris* [8].

The connecting peptide [DdPFK-(371–435)] is an essential part of the N-terminus. The most probable cause for the lack of production of an N-terminal protein functionally active *in vivo* when the connecting peptide was deleted is that an α-helix which was shown to be essential for the stability of the bacterial enzyme [31] and PFK-M (a mutation in PFK-M from dogs disrupted this helix) [32], and which is apparently conserved in DdPFK, was eliminated by either partial or complete deletion of the connecting peptide. N- (1–370) could not be complemented by co-transfection with the C-terminus with added peptide (pC-term). The simplest explanation would be inadequate folding of N-(1–370) so that it could not interact with pC-term. However, co-expression of N- (1–370) together with C-term eliminated C-terminal protein that was observed when C-term was expressed alone. This suggests some sort of association of the two subunits in a conformation recognized as damaged, such that both are then degraded, perhaps through joint ubiquitination or acetylation.

In conclusion, the present study shows the distinct roles of the two terminal halves of eukaryotic PFK. The N-terminal half contains the catalytic site, in agreement with the higher similarity of this region to the prokaryotic enzyme [3]. The evolutionarily added C-terminal half stabilizes the enzyme by promoting formation of the eukaryotic tetrameric structure, in addition to accommodating extra metabolite binding sites for the more complex regulation in eukaryotic cells.

**AUTHOR CONTRIBUTION**

Oscar H. Martínez-Costa, Valentina Sánchez, Antonio Lázaro and Eloy D. Hernández carried out the experiments. Oscar H. Martínez-Costa and Juan J. Aragón designed the research. Oscar H. Martínez-Costa, Keith Tornehmen and Juan J. Aragón contributed to data analyses and writing the paper.

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SUPPLEMENTARY ONLINE DATA

Distinct functional roles of the two terminal halves of eukaryotic phosphofructokinase

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Figure S1 Time course of the PFK reaction in cell-free extracts from yeast transformants expressing either the N-terminal half or the wild-type form of DdPFK and HmPFK-M

Extracts were prepared similarly in all strains in 50 mM Tris phosphate, 50 mM sodium fluoride, 0.1 mM sodium EDTA, 1 mM dithiothreitol, 0.5 mM PMSF and 1× protease inhibitor cocktail (Roche) (pH 8). The reaction rate of the N-terminal halves diminished gradually upon addition of substrate, reaching the basal ratio (without Fru-6-P) after 1.5 and 3 min for the N-terminus of HmPFK-M and DdPFK respectively. Extracts obtained in buffer containing 6 mM Fru-6-P showed no difference in N-terminal stability. Inset, specific activity of the N-terminal halves of HmPFK-M and DdPFK and the corresponding wild-type enzymes. Activities of the N-terminal halves were measured during less than the initial 1st min. Initial velocities of DdPFK and HmPFK-M were taken from the linear phase of the reaction. Reaction conditions for the HmPFK-M series were 6 mM Fru-6-P, 1 mM MgATP (pH 8.2), 100 mM potassium phosphate, 20 mM (NH₄)₂SO₄ and 2 mM cAMP. The extract volumes used were 2 μl of HmPFK-M and 50 μl of N-term HmPFK-M. The activity of N-term HmPFK-M was 0.4% of that with the wild-type enzyme (inset) and was not modified by the addition of either 10% poly(ethylene glycol) 6000 or 10 μM Fru-2,6-P₂, (assayed under both of the conditions above, and at 2 mM Fru-6-P and 1 mM MgATP (pH 7.2) in the absence of P₇, NH₄⁺ and cAMP). Reaction conditions for the DdPFK series were 1 mM Fru-6-P and 1 mM MgATP (pH 7.2). The extract volumes used were 2 μl of DdPFK and 20 μl of N-term DdPFK. N-term activity was approximately 0.7% of that with the wild-type enzyme (inset).

Figure S2 Co-expression of the two terminal halves of PFK improves growth of transformants

Growth of the strain HD152-1D transformed with plasmids carrying the sequence of either the N-terminal half (△) or wild-type DdPFK (○), or co-transformed with both plasmids each carrying the coding sequence of either the N-terminal or the C-terminal half of this enzyme (●). Cells were grown on YNB medium with 2% (w/v) glucose and the appropriate auxotrophic requirements. Inset, generation time of transformants expressing the indicated recombinant proteins. Values are means ± S.E.M. for three to five determinations of three independent experiments.

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Figure S3 Western blot analysis of a chimaeric form of DdPFK and HmPFK-M

Blots of cell extracts from transformants expressing either HmPFK-M, DdPFK, ChiDdM–PFK or the N-terminal half of DdPFK were probed with the antibodies indicated. Recognition of ChiDdM–PFK by antibodies that were specific for the donating enzymes indicated its chimaeric nature, showing a band of 92 kDa, which agrees with the calculated molecular mass from the protein sequence. The protein corresponding to the N-terminus of DdPFK was detected as a major band of 48 kDa in the blot probed with anti-DdPFK (see also Figures 3A and 5B of the main paper). Other minor bands present in the latter blot reflect the relatively lower specificity of the antibody. Positions of molecular mass markers are shown on the right.

Figure S4 Size-exclusion FPLC of chimaeric ChiDdM–PFK

A cell extract from transformants expressing ChiDdM–PFK was chromatographed on a Superdex 200HR 10/30 column. Arrows indicate the theoretical elution volumes of ChiDdM–PFK forms corresponding to tetramer, dimer and monomer and their calculated molecular mass values in kDa. The chromatographic profile of DdPFK (from Figure 3 of the main paper) is shown as a broken line for comparison. ChiDdM–PFK eluted in three peaks. Of this, 26% of the eluted protein was calculated to be in a tetrameric form (second peak), the remaining protein either aggregated into forms larger than tetramers (first peak) or dissociated to monomers (third peak).

Table S1 Sequence of the mutagenic primers used in the present study

The endonuclease restriction sites introduced are underlined. Initiating and stop codons are in bold.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Synthetic oligodeoxynucleotides (5′→3′)</th>
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<tbody>
<tr>
<td>Dd-Nterm</td>
<td>CCGCATTGCTCACTGTGTGTCCTCTGCTCTCAAC</td>
</tr>
<tr>
<td>Dd-Cterm</td>
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<tr>
<td>M-Cterm</td>
<td>CCAGATCTACACACAGTGGCTGTGATGAAC</td>
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