Inhibition of triosephosphate isomerase by phosphoenolpyruvate in the feedback-regulation of glycolysis

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1. Summary

The inhibition of triosephosphate isomerase (TPI) in glycolysis by the pyruvate kinase (PK) substrate phosphoenolpyruvate (PEP) results in a newly discovered feedback loop that counters oxidative stress in cancer and actively respiring cells. The mechanism underlying this inhibition is illuminated by the co-crystal structure of TPI with bound PEP at 1.6 Å resolution, and by mutational studies guided by the crystallographic results. PEP is bound to the catalytic pocket of TPI and occludes substrate, which accounts for the observation that PEP competitively inhibits the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Replacing an isoleucine residue located in the catalytic pocket of TPI with valine or threonine altered binding of substrates and PEP, reducing TPI activity in vitro and in vivo. Confirming a TPI-mediated activation of the pentose phosphate pathway (PPP), transgenic yeast cells expressing these TPI mutations accumulate greater levels of PPP intermediates and have altered stress resistance, mimicking the activation of the PK–TPI feedback loop. These results support a model in which glycolytic regulation requires direct catalytic inhibition of TPI by the pyruvate kinase substrate PEP, mediating a protective metabolic self-reconfiguration of central metabolism under conditions of oxidative stress.

2. Introduction

With the challenge of surviving in a constantly changing environment, cells have evolved mechanisms to flexibly regulate metabolism [1,2]. An important and dynamically regulated metabolic pathway is glycolysis, an ancient chemical route of carbohydrate utilization that produces ATP, NADH and intermediate metabolites for the synthesis of nucleotides, fatty acids and amino acids. Glycolysis is mainly regulated through feedback and feed-forward cycles involving its intermediate metabolites. These cycles sustain intermediates while preventing their accumulation to toxic levels and are responsible for the oscillating behaviour of glycolytic reactions [3–6]. Moreover, this enzymatic regulation is important for maintaining the balance of metabolism during changes in cell growth or environment [1,2]. As an example, the increased need for the redox cofactor NADPH during oxidative stress caused upon hydroperoxide exposure is met by diverting...
glycolytic flux into the pentose phosphate pathway (PPP). This transition is rapidly inducible by metabolic inhibition of glycolysis, changes in the activity of glucose 6-phosphate dehydrogenase (the first enzyme of the oxidative PPP), followed by transcriptional control during mid- to long-term adaptation to oxidative conditions [7–10].

A similar mechanism acts to prevent an accumulation of oxidizing metabolites in cancer cells or cells that respire at high rates. These frequently possess a higher activity of the PPP to balance the greater demand for NADPH by the antioxidant machinery and to compensate for the increased production of reactive oxygen species [11,12]. Current findings have highlighted the importance of the terminal glycolytic enzyme pyruvate kinase (PK) to achieve the regulation of glycolysis and the PPP. Low activity of PK has been found in cancer and rapidly proliferating cells, and in yeast cells with high respiration activity [13,14]. More recently, it has been proposed that cancer cells profit from the loss of the PKM2 gene during tumour formation [15]. Reduced PK activity caused accumulation of its substrate, phosphoenolpyruvate (PEP), which correlates with an increased activity of the PPP [14,16], and increased oxidant tolerances of both mammalian and yeast cells [11,14]. It has been observed that PEP is an inhibitor of another metabolic redox regulator, triosephosphate isomerase (TPI or TIM, EC 5.3.1.1) [17,18]. In its glycolytic role, TPI is regarded as a near-perfect catalyst because its catalytic speed (TPI or TIM, EC 5.3.1.1) [17,18]. In its glycolytic role, TPI is interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) to prevent an accumulation of DHAP [19,20]. Reduced activity of TPI in yeast and Caenorhabditis elegans leads to a partial inhibition of glycolysis but is beneficial during oxidative stress, as it increases the concentration of PPP metabolites and stress tolerance in both species [7,21]. We have shown previously that the increased oxidative stress resistance of PK mutants is attributable to TPI as well. In yeast cells expressing mutant TPI with lowered activity, PK failed to increase stress resistance, while a deletion of the first enzyme of the oxidative PPP, glucose 6-phosphate dehydrogenase (G6PDH, ZWF1), leads to protein and mitochondrial oxidative damage in a PK-dependent manner [14].

To understand how PEP affects TPI activity, we generated a co-crystal structure of the enzyme in complex with PEP at 1.6 Å resolution. We find that PEP directly interacts with TPI by binding into the catalytic pocket of the enzyme and outcompetes the substrates from their binding position. Moreover, the structural data reveal that PEP interacts with the conserved Ile170, a residue which when mutated is associated with TPI deficiency in humans [22], and in yeast affects response to oxidative stress [7,23] and PK function [14]. We use this mutant and others inferred from the crystallographic structure to define the kinetics and stability properties of TPI upon PEP binding. We demonstrate that the in vivo consequence of competitive TPI inhibition is the activation of the PPP and altered stress resistance.

3. Results and discussion

3.1. Structure of the triosephosphate isomerase–phosphoenolpyruvate complex

TPI is a ubiquitous enzyme with homologues found throughout all kingdoms of life [20,24] and that in human populations possesses only a minimum of sequence divergence [25]. To study the TPI–PEP interaction, we co-crystallized PEP and rabbit TPI, which differs from human TPI in four non-conserved residues only (electronic supplementary material, figure S1). The structure was solved by molecular replacement and refined at 1.55 Å resolution (table 1). The asymmetric unit contains a homodimer of TPI (figure 1). Each protomer contains eight a-b-strands on the outside and eight parallel a-helices on the inside, forming a typical TIM-barrel [26]. Comparison of TPI–PEP with a previously reported structure of rabbit muscle apo TPI [27] shows that the active site loops are in the closed conformation in both subunits. The electron density map gave a clearly defined and unambiguous shape for PEP bound to the active sites of both subunits (figure 1a,c). Active site residues engage PEP and make similar interactions to those observed for the TPI substrate DHAP (figures 1b and 2a,b); a stereo-scopic illustration is given in figure 1c) [28]. For substrate conversion, TPI employs a catalytic triad consisting of the residues Lys13, His95 and Glu165 [27], whereas PEP is in contact with the catalytically active residue Lys13 and the active site residues Gly323, Gly233, Gly171, Ser211 and Asn11 (figure 2). The positioning of PEP thus indicates that it binds into the

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purified the enzymes using metal affinity chromatography. Far-UV circular dichroism (CD) spectroscopy of the purified recombinant enzymes showed a similar composition of secondary structures, indicating that the mutations did not prevent folding (electronic supplementary material, figure S2). To determine the impact of the mutations on the interactions of TPI with PEP and G3P, we conducted thermal melt assays using the fluorescent probe SYPRO Orange [30]. In the presence of incremental PEP concentrations, TPI and its mutants exhibited thermo stabilization, indicating that the proteins bound the metabolite (figure 3a). Interestingly, TPI_{Ile170Val} and TPI_{Ile170Thr} responded more strongly to the presence of PEP (TPI_{Ile170Val} ΔT_m = 2.64 °C, TPI_{Ile170Thr} ΔT_m = 2.95 °C) in comparison with a ΔT_m of 2.57 °C for human wild-type TPI, indicating that the mutations increased the binding affinity to PEP (figure 3a).

Next, we assessed structural stability in the presence of the TPI substrate, G3P. This substrate is expected to be constantly metabolized to DHAP (and back) [31,32], and adding up to 3 mM G3P to wild-type TPI caused a slight increase in the enzyme’s thermal stability (figure 3b). The effects of G3P addition to TPI_{Ile170Val} were comparable with that of the wild-type. A much stronger response was however observed for TPI_{Ile170Thr}. This mutant substantially gained stability in the presence of G3P (figure 3b; ΔT_m = 6.21 °C at 3 mM G3P, wild-type TPI ΔT_m = 1.68 °C), indicating that the binding affinity to this substrate was increased. We speculate that the increased substrate affinity is facilitated by a hydrogen bond between the substrate and the threonine side chain.

Finally, we tested whether protein stability is affected by PEP in the presence of G3P. In the wild-type form, PEP was competitive with G3P for binding the enzyme, as expressed by an increase in thermal stability even at PEP levels lower than 0.25 mM (figure 3c). Conversely, the increased thermostability mediated by G3P specifically to the TPI_{Ile170Thr} enzyme (figure 3b) was partially lost upon adding PEP (figure 3c), confirming competitive binding in this mutant as well. By contrast, TPI_{Ile170Val} was resistant to increased PEP levels (figure 3c), indicating that this metabolite was no longer competitive for binding. In summary, thermal shift assays confirmed binding of PEP to TPI. The different behaviour of the TPI_{Ile170Val} and TPI_{Ile170Thr} mutants in this process supports the crystallographic identification of the binding site to be the catalytic pocket and indicates direct contact of PEP and G3P with this isoleucine residue.

### 3.2. Structure–function analysis of the triosephosphate isomerase–phosphoenolpyruvate interaction

We observed that PEP is in direct contact with a conserved isoleucine at position 170. A human TPI allele mutant for this residue (Ile170Val) has been found in a rare variant in the human genetic disorder TPI deficiency. This mutation translates into a mutant TPI with reduced catalytic activity [21,22]. TPI deficiency manifests as recessive autosomal multi-system disorder, which is caused by structural defects in the TPI enzyme [29]. Based on the crystallographic information, we predicted two further residue exchanges to affect PEP binding and generated two constructs encoding for TPI_{Lys13Arg} as well as TPI_{Ile170Val}.

Lys13 is known to be required for the catalytic mechanism [26] and exchanging it to arginine rendered the enzyme not only catalytically inactive but also largely unstable (electronic supplementary material, figure S3; figures 4 and 5). Thus, our analyses shown below focused mostly on the TPI_{Ile170Val} and TPI_{Ile170Thr} proteins that retained stability and residual catalytic activity.

#### 3.3. TPI_{Ile170Val} and TPI_{Ile170Thr} exhibit altered phosphoenolpyruvate and glyceraldehyde-3-phosphate binding

We expressed 6x-histidine tagged wild-type human TPI, TPI_{Ile170Val}, TPI_{Ile170Thr} and TPI_{Lys13Arg} in Escherichia coli and

![Figure 1. Co-crystal structure of TPI with bound PEP. (a) Schematic of the TPI–PEP crystallographic structure. PEP locates in the active centre of both subunits in the asymmetric TPI dimer. (b) The catalytic pocket of TPI bound to PEP. Catalytic residues are highlighted in yellow, PEP in red, isoleucine 170 in green. (c) Stereoscopic illustration of the PEP binding site environment including a difference map in which PEP has been removed from the model and was refined against the experimental data for five cycles. The map has been contoured at 4 s.d. and reveals positive density for the missing ligand.](http://rsob.royalsocietypublishing.org/content/10/4/130232)
saturation and PEP titration curves for these enzyme species. The mutant enzymes exhibited lower substrate conversion rates and saturated at lower concentrations of G3P ($K_m$ for wild-type TPI: 1373 μM, TPIIle170Val: 687 μM and TPIIle170Thr: 303 μM). This indicates that despite its lower activity, the TPIIle170Thr mutant had higher affinity to the TPI substrate. (figure 4b, black curves, from left to right). Next, we titrated PEP to the reaction operating at maximal activity. In all cases, a strong and concentration-dependent inhibition of the enzyme activity was observed. In the case of human wild-type TPI, 50% of enzyme activity was lost in the presence of 570 μM PEP (IC50), corresponding to a $K_i$ of 230 μM.

Figure 2. The TPI inhibitor PEP and the TPI substrate DHAP have similar interaction sites. (a) Contact distances between TPI and its substrate DHAP, and (b) the interactions of TPI and PEP in the active site. PEP and DHAP are in contact with similar principal residues. Distances are given in Å. Green balls, phosphate; grey balls, carbon; red balls, oxygen. The red circles indicate residues in close proximity to the ligand. Illustrations were prepared using LuProt. (c) PEP and DHAP bind similarly to the TPI active site. Rabbit TPI bound to PEP, overlaid with the location of the TPI substrate DHAP as determined by Jogl et al. [28] as surface representation. Yellow areas highlight catalytically active residues; PEP: red; DHAP: blue.

Figure 3. PEP competes with G3P for binding to human TPI. (a) Thermal stability of human TPI and active-site mutants TPIIle170Val and TPIIle170Thr in the presence of increasing PEP concentrations. PEP stabilized the three-enzyme species indicative for binding; TPIIle170Val and TPIIle170Thr were stabilized to an increased extent. (b) Thermal stability of human TPI mutants to increasing G3P concentrations; increased thermal stability of TPIIle170Thr indicated augmented affinity for G3P. (c) PEP dose–response curve in the presence of G3P. PEP binding was competitive against G3P in human TPI and TPIIle170Thr but did not influence the thermal stability of TPIIle170Val.
Figure 4. PEP inhibits the catalytic activity of TPI. (a) TPI_{ile170val} and TPI_{ile170thr} have reduced catalytic activity, TPI_{lys13arg} is inactive. Enzyme activity expressed as substrate conversion rate in micromoles per minute and microgram protein. (b) Enzymatic properties of TPI, TPI_{ile170val}, TPI_{ile170thr} and their inhibition by PEP. (c) Substrate titration curves of G3P (black curves, to be read from left to right) on TPI and its mutant enzymes, as well as inhibitor titration curves for PEP (blue curves, to be read from right to left). Substrate/inhibitor saturation was used to calculate $V_{\text{max}}$, $K_m$ (G3P titrations), and IC$_{50}$ and $K_i$ values (PEP titrations) (inset table).

Figure 5. Human TPI_{ile170val} and TPI_{ile170thr} complement for yeast TPI and are catalytically active. (a) TPI, TPI_{ile170val} and TPI_{ile170thr}, but not TPI_{lys13arg}, complement for yeast TPI$_1$. In a plasmid shuffle experiment, Δtpi1 cells carrying a counterselectable TPI-encoding plasmid were transformed with a centromeric plasmid (mini-chromosome) encoding the indicated TPI mutants. Transformed cells were then transferred to 5'FOA to induce loss of the counterselectable plasmid. Only cells containing a functional TPI copy on the minichromosome are viable on glucose media after counterselection. Human TPI, TPI_{ile170val} and TPI_{ile170thr} complemented for a loss of the TPI plasmid, but TPI_{lys13arg} did not. (b) TPI activity in yeast whole-cell extracts. Substrate conversion rates as normalized to total protein content. TPI_{ile170val} and TPI_{ile170thr} have lower activity than wild-type TPI. (c) Increased expression levels of TPI_{ile170val} and TPI_{ile170thr} in yeast as revealed by immunoblotting of whole-cell extracts using a TPI-specific antibody [35]. The amount loaded onto the SDS-PAGE gel was normalized to total protein, comparable loading was evaluated by Ponceau Red staining of the blotting membrane.
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genic strains. As the total TPI substrate conversion per 
sing wild-type TPI, TPI Ile170Val and TPIIle170Thr could be 
catalysing the same reaction, and which is kept viable by 
expressing TPI from a 5'FOA-counterselectable URA3 plasmid. We introduced human TPI, TPIIle170Val, TPIIle170Thr and 
TPILys13Arg into this strain, then selected on 5'FOA media for 
cells that had lost the TPI-URA3 plasmid. Yeast strains expressing 
wild-type TPI, TPIIle170Val and TPIIle170Thr could be 
cultured in glucose-containing media, indicating that these 
enzymes compensated for the loss of yeast TPI, demonstrating 
catalytic activity in vitro. By contrast, yeast cells expressing 
TPILys13Arg were not viable, confirming that TPILys13Arg was not catalytically functional (figure 5d).

Next, TPI activity was measured in cell extracts of the trans-
genic strains. As the total TPI substrate conversion per 
microgram protein in the cell extract corresponded to 1.5% com-
pared to the pure enzyme (15.5 μmol NADH min⁻¹ μg protein⁻¹), we estimate that TPI accounts for approximately 1.5% of total soluble protein, substantiating that TPI is one of the most abundant cytoplasmic proteins [34]. Interestingly, we noted that the total activity of mutant enzymes (TPIIle170Val, 
TPIIle170Thr) was, relative to wild-type, significantly lower in 
their purified version compared with what we measured in 
the cell extracts (figures 4a and 5b). An analysis of TPI 
expression levels by immunoblotting using a specific TPI anti-
sera [35] however revealed that mutant TPI is much more 
strongly expressed compared with wild-type TPI (figure 5c). This indicates that cells compensated for a loss of specific TPI activity by the upregulation of the enzyme abundance.

3.6. Low triosephosphate isomerase activity mediates elevation in pentose phosphate pathway metabolite concentrations, oxidant resistance and heat sensitivity

We have shown previously that reduced activity of TPI causes a re-configuration of central metabolism, leading to increased flux of the PPP and increased stress resistance in yeast and C. elegans [7]. The feedback inhibition of TPI by PEP is therefore expected to have similar consequences. In bacteria, yeast and mammalian cells, PEP accumulation is caused by a diminution of PK activity [11,14,16]. Whereas low PK activity in yeast is correlated with high respiration rates and superoxide production [14], in 

human cells it is associated with rapid cell proliferation and cancer [13,36]. Affected by high ROS production, cancer cells upregulate the allosterically regulated PK isoform PKM2 [37], which is redox-sensitive and the PKM isoform with lower catalytic activity [11,13,36]. Moreover, recent results have demonstrated that cancer cells have higher survival chances when they lose this gene [15]. This situation causes a block of the early steps of glycolysis and increases the PPP activity resulting in augmented oxidant tolerance of both yeast and mammalian cells [11,14], indicating that the PK–TPI feedback loop is important for oxidative stress protection.

As shown above, TPI substrates and PEP bind to the same 
structural site and have largely the same contact residues. As a consequence, mutations that affect PEP binding also affect the catalytic activity of TPI. This prevents the creation of an ideal in vivo model where TPI feedback inhibition by PEP would be disrupted while TPI catalytic activity is unaffected. However, the mutant proteins provide a means of studying the consequences of specifically lowered TPI activity that mimics the situation of feedback inhibition. We used the yeast strains expressing 
TPIIle170Val and TPIIle170Thr to determine glycolytic and PPP metabolite concentrations by liquid chromatography tandem mass spectrometry (LC-MS/MS), adapting our previous procedures [38,39]. In comparison with the isogenic strain 
expressing wild-type TPI, yeast cells expressing both the naturally occurring TPIIle170Val allele and the designed TPIIle170Thr protein displayed an increased concentration of PPP intermediates, indicating higher activity of this pathway confirming the previous results (figure 6a; electronic supplementary material, figure S4). Moreover, glycolytic intermediates upstream of TPI were affected, with the strongest measured increase in the concentration of the TPI substrate DHAP (figure 6a), reflecting the partial blockage of glycolysis.

Next, we tested for consequences of expressing the mutant 
TPI forms in regard to stress resistance. For this, the transgenic 
strains were rendered prototrophic by transformation with the pHILUM minichromosome [40]. Then, the cells were spotted on 
media containing the thiol-oxidizing compound diamide, as 
resistance to this compound has previously been shown to be 
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Exponentially growing yeast strains were exposed to 50°C for five minutes or kept at 30°C and used to inoculate a fresh culture. The heat-induced growth delay, calculated using a model free spline fit [45], was used as a measure of yeast heat resistance. Yeast cells expressing human TPI well tolerated the heat treatment; however, yeast harbouring TPI\textsubscript{Ile170Val} and TPI\textsubscript{Ile170Thr} were heat-sensitive, resulting in a strong delay until growth resumed (figure 6c). Thus, low TPI activity, despite protecting against oxidants, causes heat sensitivity. In summary, similar to what has been observed

\[\text{Figure 6.} \text{ Low TPI activity increases PPP metabolite load and causes oxidant resistance and heat sensitivity. (a) Concentrations of glycolytic and PPP metabolites in the human TPI\textsubscript{Ile170Val} and TPI\textsubscript{Ile170Thr} mutants relative to yeast expressing human wild-type TPI. PPP and glycolytic metabolites were quantified by LC-MS/MS. PPP metabolites are increased in the TPI mutants. Absolute values are given in the electronic supplementary material, figure S4. (b) TPI\textsubscript{Ile170Val} and TPI\textsubscript{Ile170Thr} mediate increased tolerance to oxidizing agents. Overnight cultures of the indicated yeast strains were diluted to an OD\textsubscript{600} = 3 and spotted onto SC\textsuperscript{2}His agar plates containing the oxidants. Glucose 6-phosphate dehydrogenase (Zwf1) encodes the enzyme for the first step in the non-reversible oxidative PPP shunt and produces NADPH. Its deletion abolishes the oxidant resistance phenotype of cells expressing TPI\textsubscript{Ile170Val} or TPI\textsubscript{Ile170Thr}. Sol3 and Sol4 catalyse the second step of the PPP and their deletion reduced oxidant resistance on H\textsubscript{2}O\textsubscript{2}; a protective effect of TPI\textsubscript{Ile170Val} was detected in D\textsubscript{sol3} yeast while causing H\textsubscript{2}O\textsubscript{2} sensitivity in D\textsubscript{sol4} yeast. (c) TPI mutants are heat-sensitive. Overnight cultures were diluted to an OD\textsubscript{600} = 0.2 and exposed, or not exposed, to 50°C for 5 min and growth was monitored for 25 h after heat exposure. The duration until growth was re-established (lag phase) was used as an inverse indicator for heat resistance. The lag phase was prolonged in the 50°C exposed TPI mutants compared with isogenic yeast cells expressing wild-type TPI.} \]
Table 2. Plasmids used in this study and their deposition ID (http://www.addgene.org).

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in cells with low PK activity [14,44], expressing TPIile170Val and TPIile170Thr increased PPP metabolite concentrations and mediated oxidant resistance and heat sensitivity.

4. Conclusion

The central glycolytic enzyme TPI plays a crucial role in coordinating energy with redox metabolism during stress response and in cancer. Being the target of a feedback loop initiated by the pyruvate kinase substrate PEP, dynamic TPI inhibition distributes metabolites between glycolysis and the PPP [7,14]. Here we present a TPI–PEP co-crystal structure, demonstrating that PEP directly binds into the catalytic pocket of TPI. In structure–function studies involving different TPI point mutations including a rare natural variant (TPIIle170Val [22]), and two mutants designed on the basis of the crystallographic findings (TPILys13Arg and TPIile170Thr), we have demonstrated that PEP functions as a competitive TPI inhibitor, being able to interfere with the enzymatic TPI function during catalysis. Finally, studies with transgenic yeast cells expressing these human TPI mutants revealed that low TPI activity increases PPP metabolite concentrations, increased oxidant resistance and decreased heat tolerance. Hence, the PYK–TPI feedback loop, leading to the regulation of glycolysis and the PPP to adapt to oxidative stress conditions, is the consequence of active-site competitive TPI inhibition by the PK substrate PEP.

5. Material and methods

Recombinant TPI expression, enzyme purification, Western blotting, yeast cultivation and strain generation were conducted according to standard procedures and are described in the electronic supplementary material. The plasmids generated in this study have been deposited at Addgene (http://www.addgene.org) and are listed in table 2.

5.1. Crystallization of triosephosphate isomerase–phosphoenolpyruvate complex

Native rabbit muscle TPI (TPI, Sigma) was buffer exchanged into crystallization buffer (20 mM Tris pH 7.0, 150 mM NaCl, 5 mM MgCl₂) with a HiTrap Desalting column and concentrated to 10 mg ml⁻¹ with a VIVA spin 2 ml concentrator (MWCO: 10 kDa). PEP was added to the TPI solution to a final concentration of 5 mM. Crystals were grown at 20°C using the sitting-droplet vapour diffusion method by mixing 200 nl of TPI–PEP complex with 200 nl of reservoir solution (0.1 M MES pH: 6.5, 25% polyethylene glycol (PEG) 8000). Crystals appeared 1 day after setting up the crystallization trial and reached the final size in 1 week. The crystals were transferred briefly into reservoir solution supplemented with 25% v/v PEG 400 as cryoprotectant before flash freezing in liquid nitrogen.

5.2. Data collection, structure determination and refinement

X-ray diffraction data were collected at 100 K from cryoprotected crystals at beamline I24 at the Diamond Light Source. A complete dataset of TPI–PEP crystal was collected to a resolution of 1.55 Å. The data were processed and scaled using iMOSFLM and SCALA [46,47], respectively. Molecular replacement was performed with the CCP4 suite program Phaser [48] using the rabbit muscle apo TPI (PDB ID: 1R2R) [27] as the search model. The map identified PEP in the active site, and the initial model (without ligand) was refined using Refmac5 [49]. One protomer was manually adjusted into the electron density map using COOT and directly placed in the second protomer based on non-crystallographic symmetry. The model was refined again with TLS, NCS (non-crystallographic symmetry) and restrained refinement using Refmac5. PEP was finally built into the electron density map and then refined. A summary of the crystallographic data and refinement are given in table 1. Figures were generated using PYMOL.

5.3. Circular dichroism

Recordings of the far-ultraviolet (UV) CD spectrum were used to verify the native conformation of the purified TPI enzyme species. The TPI proteins were diluted to a final concentration of approximately 3.7 mM in 20 mM HEPES (pH 7.5). CD recordings were performed at 25°C on a Jobin Yvon CD6 Dichrograph, as described previously [50]. Three scans were averaged and base line subtracted using the software provided by the Jobin Yvon CD6 Dichrograph manufacturer.

5.4. Enzyme activity assays

TPI activity was determined as described previously [21,51]. In brief, activity of TPI in cell-free protein extracts of

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transgenic yeast expressing human TPI, or with purified human TPI recombinantly expressed in E. coli, was determined in an enzyme-coupled reaction with glycerol 3-phosphate dehydrogenase. Optical density measurements at 340 nm were used to detect NADH to NAD+ oxidation upon adding the TPI substrate glyceraldehyde 3-phosphate and recorded in 12 s intervals in a spectrophotometer (Amersham US 2000). $K_m$ and $K_i$ values were determined by generating saturation curves with G3P and PEP, respectively.

5.5. Thermal shift assays

The detection of protein thermal unfolding was performed in 96-well plates on an iQ5 real-time PCR cycler (BioRad). The reaction mix of 100 ml 20 mM HEPES (pH 7.5) contained approximately 4.5 mM protein, 0.4 ml 50% SYPRO Orange (Sigma Aldrich) and PEP and/or G3P at the indicated concentrations. Thermal unfolding of the proteins was monitored by increasing the temperature from 25 to 95 °C in 2 °C min⁻¹ steps. Measurements were taken every 0.5 °C. The resulting curves were each fitted with a four-parameter log-logistic function and protein melt points (inflection points) were calculated using R v. 2.14.1 and the drc package v. 2.3-0. The protein melt points for each ligand concentration were plotted against the melt temperature and fitted with equation (5.1).

$$y = \frac{T_{\text{max}} \times x}{K_d + x} + a \times x + b. \quad (5.1)$$

5.6. Oxidant-tolerance tests and growth curves

Oxidant tolerance tests were performed as described earlier [7] and growth was monitored after 2–3 days of incubation at 30 °C. For growth curves, overnight cultures of the indicated yeast strains were diluted to an OD600 of 0.2 in SC−His media. Aliquots of the same cultures were incubated for 8 concentrations. Thermal unfolding of the proteins was monitored by 12% acetonitrile for 3.5 min followed by a gradient to 38% acetonitrile within 2.5 min. With an additional washing step (42% acetonitrile, 0.5 min) and re-equilibration to starting conditions, this resulted in a total cycle time of 7.5 min. All buffers contained 750 mg l⁻¹ octylammoniumacetate as ion pairing reagent. An online coupled triple quadrupole mass spectrometer (Agilent 6460) operating in SRM mode was used for quantification. Individual metabolites were identified by matching retention time and fragmentation pattern with commercially available standards. SRM transitions, ionization and fragmentation energies were optimized for each compound (electronic supplementary material, table S2). Ion source settings are listed in the electronic supplementary material, table S3. Data analysis was done in the Masshunter Workstation software package (Agilent). External calibration curves were measured repeatedly and used to determine absolute concentrations.

Acknowledgements. We thank our laboratory members for critically reading and discussing the manuscript and Dr Katherine Stott (University of Cambridge, UK) for technical support. M.R. is a Wellcome Trust Research Career Development and Wellcome-Beit prize fellow.

Data accessibility. The coordinates of the PEP-bound TPI crystallographic structure have been deposited with the PDB with code 4QWG.

Funding statement. We acknowledge funding from the Wellcome Trust (RG 093735/Z/10/Z) and the ERC (Starting grant 260809) to M.R. M.A.K. is supported by an Erwin Schrödinger postdoctoral fellowship (J 3341) from the FWF (Austria). B.F.L. and D.D. are supported by the Wellcome Trust.

References


27. Kroger A et al. 2011 The pentose phosphate pathway is a metabolic redox sensor and regulates transcription during the antioxidant response. Antioxid. Redox Signal. 15, 311 – 324. (doi:10.1089/ars.2010.3797)


