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Review

Properties and functions of the thiamin diphosphate dependent enzyme transketolase

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Abstract

This review highlights recent research on the properties and functions of the enzyme transketolase, which requires thiamin diphosphate and a divalent metal ion for its activity. The transketolase-catalysed reaction is part of the pentose phosphate pathway, where transketolase appears to control the non-oxidative branch of this pathway, although the overall flux of labelled substrates remains controversial. Yeast transketolase is one of several thiamin diphosphate dependent enzymes whose three-dimensional structures have been determined. Together with mutational analysis these structural data have led to detailed understanding of thiamin diphosphate catalysed reactions. In the homodimer transketolase the two catalytic sites, where dihydroxyethyl groups are transferred from ketose donors to aldose acceptors, are formed at the interface between the two subunits, where the thiazole and pyrimidine rings of thiamin diphosphate are bound. Transketolase is ubiquitous and more than 30 full-length sequences are known. The encoded protein sequences contain two motifs of high homology; one common to all thiamin diphosphate-dependent enzymes and the other a unique transketolase motif. All characterised transketolases have similar kinetic and physical properties, but the mammalian enzymes are more selective in substrate utilisation than the nonmammalian representatives. Since products of the transketolase-catalysed reaction serve as precursors for a number of synthetic compounds this enzyme has been exploited for industrial applications. Putative mutant forms of transketolase, once believed to predispose to disease, have not stood up to scrutiny. However, a modification of transketolase is a marker for Alzheimer's disease, and transketolase activity in erythrocytes is a measure of thiamin nutrition. The cornea contains a particularly high transketolase concentration, consistent with the proposal that pentose phosphate pathway activity has a role in the removal of light-generated radicals.

Keywords: Thiamin diphosphate; Transketolase; Pentose phosphate pathway; Kinetics; Thiamin deficiency

Abbreviations: BDC, Benzoylformate decarboxylase, DHAS, Dihydroxyacetone synthase, DHEThDP, α, β-Dihydroxyethyl-ThDP, KGDH, α-Ketoglutarate dehydrogenase, PDC, Pyruvate decarboxylase, PDH, Pyruvate dehydrogenase, POX, Pyruvate oxidase, PPP, Pentose phosphate pathway, ThDP, Thiamin diphosphate, TA, Transaldolase, TK, Transketolase, WK, Wernicke-Korsakoff.

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1. Transketolase: a thiamin diphosphate dependent enzyme

Thiamin diphosphate (ThDP) is a derivative of thiamin, the first vitamin to be isolated in pure form [71]. In 1937 Lohmann and Schuster [95] discovered that ThDP is the "active" thiamin cofactor in the enzyme pyruvate decarboxylase (PDC) from yeast. Since then ThDP has been recognised as a cofactor in several enzymes, which catalyse mainly decarboxylations of α-ketoacids or the transfer of ketol groups from donor ketoses to acceptor aldoses.

The chemical structure of ThDP is that of an aromatic methylaminopyrimidine ring, linked via a methylene bridge to a methylthiazolium ring with a pyrophosphate group attached to a hydroxyethyl side chain (Fig. 1). In non-enzymatic model studies it has been demonstrated that the thiazolium ring can catalyse reactions which are similar to those of ThDP-dependent enzymes but several orders of magnitude slower [86, 104, 185]. Using infrared and NMR spectrophotometry it has been shown that the dissociation of the proton from C2 of the thiazolium ring is necessary for catalysis; the abstraction of the proton leads to the formation of a carbanion (ylid) with the potential for a nucleophilic attack on the carbonyl group of the substrate [12–14]. It proved to be difficult to determine the pKa of the C2 atom directly, mainly because of base-catalysed reactions in water that lead to a ring-opening of thiamin [33, 62, 97]. Hopmann and Brugnoni [61] reported a pK_a of 12.7, but Bunting [15] has suggested that the actual pK_a is closer to 20, consistent with the value (16.9–18.9) reported more recently by Washabaugh and Jencks [173].

Several reviews have discussed the catalytic function of ThDP [38, 80, 136, 142, 169]. In all ThDP-dependent enzymes the abstraction of the proton from the C2 atom is the first step in catalysis, which is followed by a nucleophilic attack of this carbanion on the substrate. Subsequent cleavage of a C–C bond releases the first product with formation of a second carbanion (2-α-carbanion or enamine). The formation of this 2-α-carbanion is the second feature of ThDP catalysis common to all ThDP-dependent enzymes.

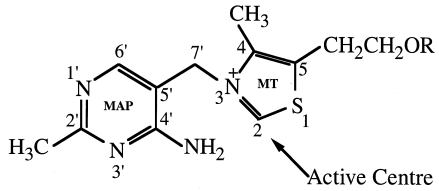

Fig. 1. Structure of thiamin and ThDP. The methylaminopyrinidine ring (MAP) is linked via a methylene bridge to the methylthiazolium ring (MT). In thiamin R is a hydrogen atom and in ThDP R is a pyrophosphate moiety ($P_2O_6^{3-}$). The arrow points towards the C2, the active centre of ThDP-catalysed reactions.

Depending on the enzyme and the substrate(s), the reaction intermediates and products differ.

A sequence motif common to all known ThDP-dependent enzymes has been suggested to represent the ThDP-binding site [52, 127]. The consensus sequence for this motif in TK (Fig. 2) begins with the highly conserved residues Gly-Asp-Gly (GDG) followed by 21 residues with less homology. The motif ends with an invariant asparagine, preceded by a stretch of several mainly hydrophobic residues. The secondary structure predicted for this motif is that the GDG sequence forms a turn that separates a β strand from an α helix approximately 20 residues in length. The conserved asparagine at the C-terminal end of this motif is predicted to form another turn that separates this α helix from another β strand. Crystal

Hsa	**GDG**ELS**E**G**S**VW**E**AMAF**A**SIYK**L**DN**LV**AIL**D**I**N**
Cpl3	**GDG**CQM**EG**VSN**E**ACSI**A**AHWG**L**GK**LI**ALY**D**D**N**
Sce	**GDG**CLQ**EG**ISS**E**ASSL**A**GHLK**L**GN**LI**AIY**D**D**N**
Eco	**GDG**CMM**EG**ISH**E**VCSL**A**GTLK**L**GK**LI**AFY**D**D**N**
Consensus[a]	**GDG**xxx**EG**xxx**E**xxxx**A**xxxx**L**xx**LV**xxx**D**x**N**
	S A **G** **I**

[a]x denotes any amino acid

Fig. 2. "ThDP-binding" motif of TKs. The sequences are taken from the multiple alignment of 22 TK sequences in Schenk et al. [138]. Only four sequences representing mammalian (*Hsa*: human [138]), plant (*Cpl3*: *Craterostigma plantagineum* [4]), fungal (*Sce*: *Saccharomyces cerevisiae* [155]) and bacterial (*Eco*: *Escherischia coli* [152]) sources have been selected for illustration. The consensus sequence based on the entire alignment in Schenk et al. [138] is shown; x can be any amino acid.

structures [3, 30, 51, 93, 108, 111] and site-directed mutagenesis studies [18, 28, 171] have revealed that selected residues within the motif anchor the diphosphate group of ThDP through the divalent cation; no motif residue interacts directly with the thiazole and pyrimidine portions of the cofactor.

The three-dimensional structures of several ThDP-dependent enzymes have been determined; these are *Saccharomyces cerevisiae* transketolase (TK) [93, 111], *Lactobacillus plantarum* pyruvate oxidase (POX) [108], *S. uvarum* pyruvate decarboxylase (PDC) [30], *S. cerevisiae* PDC [3] and *Pseudomonas putida* benzoylformate decarboxylase (BDC) [51]. Three distinct domains can be discerned in each of these structures. In PDC, POX and BDC the motif common to all ThDP-dependent enzymes is located in the C-terminal domain, but it is in the N-terminal domain of TK. Interestingly, while PDC, POX and BDC have a collinear arrangement of domains, TK seems to be a circular permutation of the others in which the first and second domains of TK correspond to the third and first domains of the three other enzymes [109]. Robinson and Chun [129] came to similar conclusions based upon a sequence alignment which included the subunits of the E_1 enzyme of the pyruvate dehydrogenase (PDH) complexes from human, *Bacillus stearothermophilus* and *Escherichia coli* sources.

The observed structural and, to a lesser extent, sequence homology as well as the similarity of the catalytic mechanism of all characterised ThDP-dependent enzymes might reflect a common ancestry [48] followed by insertions, deletions, duplications and other mutations during the course of evolution. Recently, Pohl reviewed PDC [122]. An early review of TK has been published by Racker in 1961 [125] and Kochetov (1986) has summarised structural and mechanistic aspects of TK from baker's yeast [83]. Since then structural and sequence data have greatly increased. Here we discuss the properties and functions of TK, putting recent data in context with earlier work.

2. Occurrence and properties of TK

2.1. Sources of TK

TK occurs in all organisms in which it has been investigated. The enzyme has been at least partially purified from a variety of sources including baker's yeast [25, 65, 154], *Candida utilis* [77, 79], spinach [165], pig liver [120, 147], rat liver [116], rabbit liver [98], mouse brain [8], human leukocytes [105] and human erythrocytes [1, 10, 56, 58, 135, 158, 170]. Recently, TK has been purified from *E. coli* [37, 153]. Dihydroxyacetone synthase (DHAS), a special TK present in organisms growing on methanol, has been purified from *C. boidinii* [74, 167] and from the carboxydobacterium *Acetinobacter* sp. [128], and it is likely to be present in the actinomycete *Amycolatopsis methanolica* [2].

Genes coding for DHAS from the methanol-utilising yeast *Hansenula polymorpha* [70] and TK from several mammals [1, 100, 138, 141], yeasts [69, 102, 155], bacteria [e.g. 21, 134, 152] and plants [e.g. 4] have been cloned and/or sequenced. Currently, more than 30 full length or nearly full length sequences are available from GenBank. Two genes coding for distinctly different but closely related TKs have been cloned from *S. cerevisiae* [132, 155] and *E. coli* [68, 152]; three different forms of TK have been cloned from the desiccation-tolerant plant *Craterostigma plantagineum* [4].

Most of the kinetic and physicochemical studies of TK have focussed on the enzyme from human [10, 11, 56, 149, 161, 170], *E. coli* [37, 153] and *S. cerevisiae* [10, 32, 55, 83, 112, 175–177]. The three-dimensional structure of the latter enzyme [93, 111] has been published (PDB accession code 1TRK) and crystallographic studies on the *E. coli* enzyme have commenced [94]. Homologous expression systems are established for the *S. cerevisiae* [155] and the *E. coli* [60, 153] enzyme. For human TK two laboratories have reported [140] or made use of a bacterial heterologous expression system [149, 171]. The enzymes from other sources have been less well studied but most known characteristics are generally similar between all known TKs. Nonetheless,

there are differences and some of those are discussed below.

2.2. Substrate specificity

A broad range of substrates has been reported for the TKs from yeasts, plants and bacteria. TK from *S. cerevisiae* can utilise sugars such as D-xylulose 5-phosphate, D-sedoheptulose 7-phosphate, D-fructose 6-phosphate and D-erythrulose 4-phosphate, as well as dihydroxyacetone phosphate, dihydroxyacetone and hydroxypyruvate as donors of the transferred glycolaldehyde group. Acceptor substrates include D-ribose 5-phosphate, D-glyceraldehyde 3-phosphate, D-erythrose 4-phosphate and glycolaldehyde [83, 162]. TK from spinach leaves has a substrate specificity similar to that of the *S. cerevisiae* enzyme [165] and can also catalyse the transfer of a two-carbon fragment from hydroxypyruvate to non-phosphorylated acceptor sugars. Recent findings indicate that TK purified from *E. coli* displays substrate specificities similar to those of the reported yeast and plant TKs [153]. In contrast, mammalian TKs are somewhat more specific, using only D-xylulose 5-phosphate, D-fructose 6-phosphate and D-sedoheptulose 7-phosphate as donors, and D-ribose 5-phosphate, D-erythrose 4-phosphate, D-glyceraldehyde 3-phosphate and glycolaldehyde as acceptor substrates [105, 115, 137, 170]. The K_m values for TK from different sources and for several of the main substrates are listed in Table 1. Table 2 shows K_m values for spinach TK with unphosphorylated substrates. It is apparent that the affinity of the enzyme for unphosphorylated substrates is low. This finding is consistent with the observation that the phosphate group of the substrates interacts with TK resulting in tighter binding [112]. However, the fact that spinach TK can utilise D-xylose as a substrate makes this enzyme an interesting candidate for potential use in biotransformations, although the Michaelis constant for this substrate is high (230 mM, Table 2). D-xylose occurs abundantly in nature; however, attempts to construct an industrial fermentation system for the large-scale production of ethanol from this carbohydrate have not been very successful [e.g. 168, 186]. A recombinant yeast strain expressing spinach TK may improve D-xylose fermentation significantly. Less data are available for DHAS; the enzyme from *H. polymorpha*, *C. boidinii* and *Acetinobacter* sp. displays an even wider range of substrate specificity, including all of the substrates mentioned for yeast TK, as well as formaldehyde and acetaldehyde as acceptors [70, 74, 128]. It appears [74] that the Michaelis constants for the donor (D-xylulose 5-phosphate, 1 mM) and acceptor (formaldehyde, 0.43 mM) substrates are similar to those measured for the substrates of TK (Table 1). In general, the in vivo concentration of most of the TK (and DHAS) substrates is, as far as has been determined, in the range between one and 100 μM [e.g. 184], whereas the K_m values are generally at least one order of magnitude larger (Table 1). Thus, under the physiological range of substrate concentrations TK enzyme activity will be approximately proportional to substrate concentration.

TK has a potential for use in industrial applications (see below). For an effective application

Table 1
K_m values for selected substrates for TK from various sources, representing mammalian, fungal, plant and bacterial enzymes. Data for the human enzyme were taken from Waltham [170]. All non human data are quoted from the collection of Sprenger et al. [153]

Source	Human	*S. cerevisiae*	Spinach	*E. coli*
Xylulose 5-phosphate	0.49 mM	0.21 mM	not available	0.16 mM
Ribose 5-phosphate	0.53 mM	0.4 mM	0.4 mM	1.4 mM
Fructose 6-phosphate	7 mM	1.8 mM	3.2 mM	1.1 mM
Glyceraldehyde 3-phosphate	not available	4.9 mM	not available	2.1 mM
Erythrose 4-phosphate	0.36 mM	not available	not available	0.09 mM
Hydroxy-pyruvate	no activity	33 mM	not available	18 mM

Table 2
K_m values for unphosphorylated substrates of TK from spinach leaves. Data are quoted from Villafranca and Axelrod [165]

Substrate	D-ribose	L-lyxose	L-arabinose	D-xylose
K_m	45 mM	55 mM	120 mM	230 mM

in large-scale production the conditions which may affect the reaction need to be known. Such information includes the K_m for the substrates and the inhibition constant (K_i) for each of the products. Moreover, since TK has a ping-pong kinetic mechanism [22] in which substrates can bind to the "wrong" enzyme form, there is the potential for substrate inhibition as well. Hydroxypyruvate is likely to be a preferred donor substrate because by effective removal of the first product, CO_2 (product 1 in Fig. 3), the reaction becomes essentially irreversible. The K_m and K_i values of *E. coli* TK for this substrate are 18 mM (Table 1) and 42 mM [50], respectively. In trial experiments designed to optimise the conditions for utilising TK in biotransformations glycolaldehyde was used as an acceptor substrate [50, 103], resulting in the production of L-erythrulose. The Michaelis and substrate inhibition constants for glycolaldehyde are 16 mM and 600 mM, respectively [50]. The product L-erythrulose inhibits the forward reaction only weakly (K_i of 565 mM) [50]. However, it was also reported that incubation of *E. coli* TK over a period of 8 h with glycolaldehyde at a concentration of 500 mM resulted in irreversible loss of 60% activity [103]. Thus, for continuous large-scale operation a bioreactor needs to be designed which maintains the concentrations of the reactants and products in well defined limits (e.g. [9]); in the case of the above mentioned model reaction the donor and acceptor substrate concentrations should not greatly exceed 40 mM and 100 mM, respectively [50], whereas product accumulation up to a concentration of 500 mM does not significantly interfere with the progress of the reaction.

TK, together with transaldolase (TA), catalyses reactions in the non-oxidative branch of the pentose phosphate pathway (PPP) (Fig. 4) [183]. Based mainly on carbon balance studies and on experiments involving tracing of ^{14}C labelled sugars, Williams and coworkers have proposed an alternative version of the PPP, the so called "L-type", which apparently operates in hepatocytes from rats [178]. In this type of the PPP glucose 6-phosphate also acts as an acceptor substrate for TK, resulting in the generation of an octulose [66, 179, 180]. These findings have remained controversial. Landau and Wood [91] analysed the work of Williams and collaborators in detail. While they reported several inconsistencies in Williams' interpretation of his data they also listed several other studies which do not support the existence of an "L-type" PPP (see [91] and cited literature therein). Additionally, at concentrations up to 10 mM glucose 6-phosphate does not act as a substrate for human TK purified from erythrocytes [170], whereas the concentration of glucose 6-phosphate in red blood cells is approximately 40 μM [184]. Interestingly, in the desiccation-tolerant plant *C. plantagineum* the stress-induced expression of two TK isoforms is accompanied by an accumulation of an octulose during rehydration [4]. Thus, while the alternative type of the PPP may not operate in mammalian tissues, its existence in plants cannot be ruled out.

2.3. Oligomeric structure

The functional form isolated from baker's yeast [25, 154] is a homodimer of 74 kDa subunits, each of which contains a molecule of ThDP [83]. The crystal structure of this enzyme has confirmed this observation [93, 111]. While the two cofactor binding sites are identical according to the crystal structure, it has been speculated that a conserved hydrogen bonding pattern between the two ThDP molecules may facilitate cooperative interactions between the two active sites in TK [111]. Consistent with this hypothesis kinetic studies have revealed that TK displays kinetic inequivalence of the active sites resulting in negative cooperativity with respect to ThDP binding [32, 85]. TK purified from the yeast *C. utilis* [79], rat [63], rabbit [98],

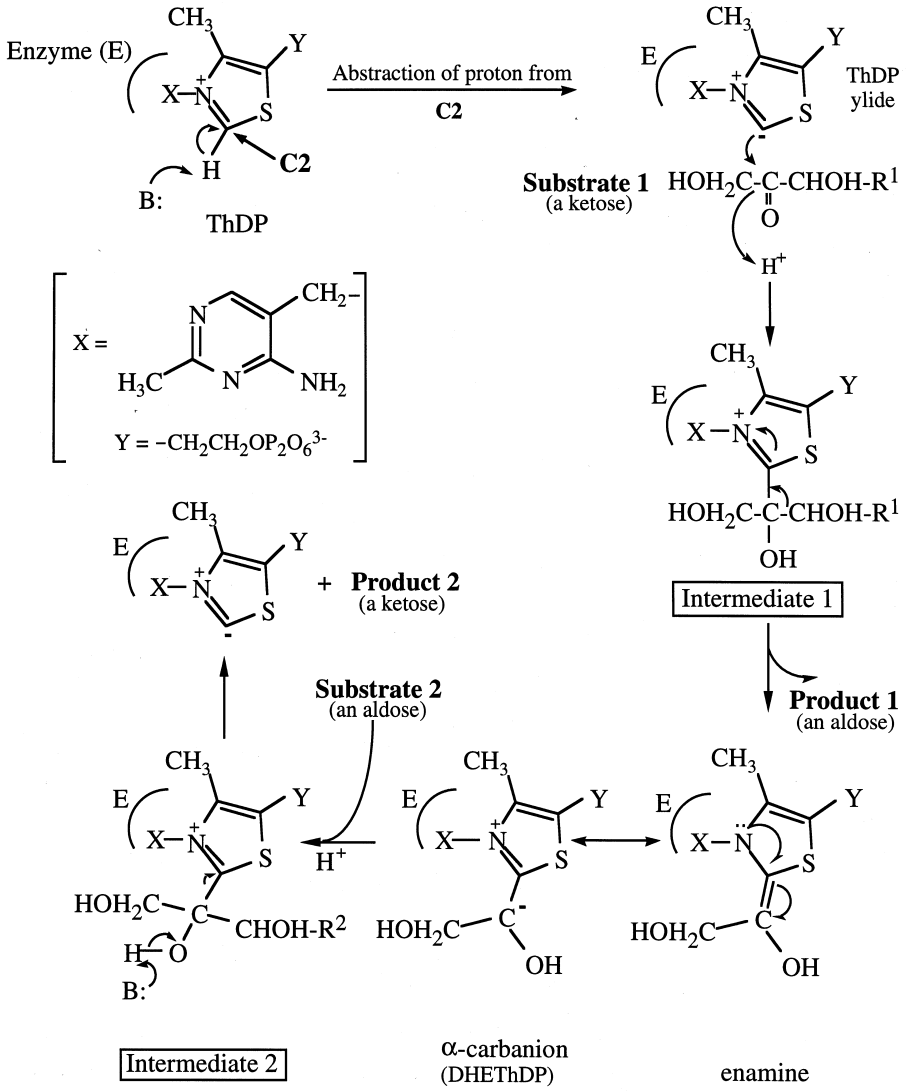

Fig. 3. Catalytic mechanism for TK. TK catalyses the transfer of the dihydroxyethyl group from a ketose donor (substrate 1) to an aldose acceptor (substrate 2). The catalytic reaction is initiated by the deprotonation of C2 of the methylthiazolium ring. This carbanion (ThDP ylide) then attacks the ketose substrate leading to the formation of the labile intermediate 1. Release of the first reaction product and tautomerisation of the resulting enamine generates a second carbanion (DHEThDP), which interacts with the aldose substrate. Release of the second product finally regenerates the active centre, which is now ready for the next cycle of catalysis.

human [56] and the plant *C. plantagineum* [4] are also homodimeric. Using gel filtration and sedimentation analysis Villafranca and Axelrod [165] demonstrated that TK extracted from spinach leaves apparently exists in monomeric form with a subunit molecular weight of approximately 100 kDa. However, the recent determination of the sequence of spinach TK [36] revealed that the subunit molecular weight is approximately 74 kDa, similar to that of other plant TKs. Moreover, a phylogenetic analysis by the same group showed that the spinach enzyme is most closely related to the constitutively expressed enzyme from *C. plantagineum* (*Cpl*3, [4]). Based

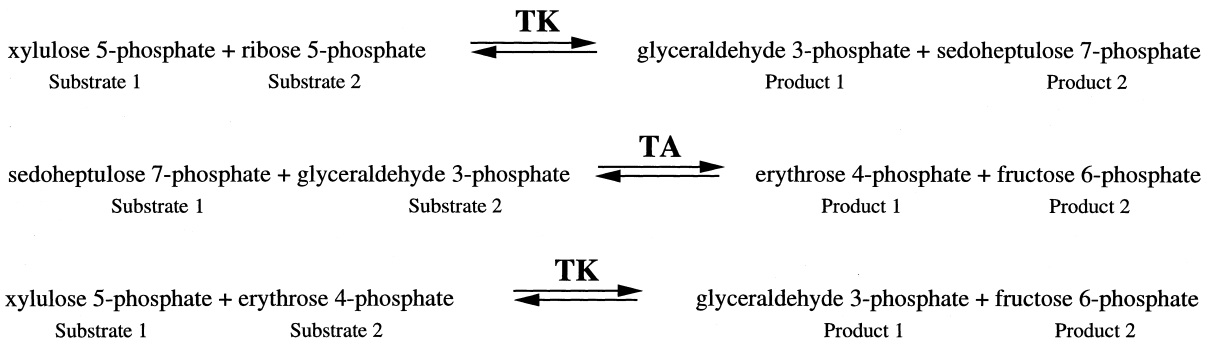

Fig. 4. Selected reactions of the PPP. TK transfers two-carbon units from donor ketoses (xylulose 5-phosphate) to acceptor aldoses (ribose 5-phosphate or erythrose 4-phosphate) resulting in the formation of aldose (glyceraldehyde 3-phosphate) and ketose (sedoheptulose 7-phosphate or fructose 6-phosphate) products. TA transfers three-carbon units from the ketose donor sedoheptulose 7-phosphate to the aldose acceptor glyceraldehyde 3-phosphate. The net result of these three reactions is the formation of one molecule of glyceraldehyde 3-phosphate and two molecules of fructose 6-phosphate from three pentoses. Subsequently, the product molecules can be further metabolised through the glycolytic pathway.

on similarities of spinach and *C. plantagineum* TKs in function and sequence it is likely that the native spinach enzyme also forms a homodimer. Treatment of a homogeneous holoTK preparation from pig liver with SDS and 2-mercaptoethanol in the presence of the protease inhibitor PMSF resulted in two bands on a polyacrylamide gel with molecular weights of 52 kDa to 56 kDa (α-subunit) and 27 kDa to 29 kDa (β-subunit) [120]. Based on these results it was concluded that native pig liver TK forms a heterotetramer ($\alpha_2\beta_2$) with a molecular weight of 138 kDa (determined by sedimentation equilibrium studies) [120]. However, incubation of the two isolated subunits in the presence of ThDP did not result in active TK holoenzyme [120]. Considering that all remaining characterised mammalian TKs are homodimers with a molecular weight of approximately 140 kDa, and all known mammalian TK sequences are highly conserved [138] it is doubtful that the pig liver enzyme differs significantly; the sequence of this TK is yet to be determined.

DHAS from *C. boidinii* is apparently a homotetramer with a subunit molecular weight of 55 kDa [74]. This is significantly smaller than that of DHAS from *H. polymorpha* [70]. Recently, DHAS has been purified from *Acetinobacter* sp. [128] using a protocol similar to that applied to the purification of this enzyme from *C. boidinii* [74]. However, the *Acetinobacter* sp. enzyme appears to be a homodimer with a subunit molecular mass of 73 kDa. At present, the variation of the oligomeric structures of *C. boidinii* DHAS on the one hand and the enzymes from *H. polymorpha* and *Acetinobacter* sp. on the other hand cannot be resolved conclusively. However, based on the results of the abovementioned studies on TK and DHAS from a broad range of sources, it appears that the general oligomeric structure of this group of enzymes is usually that of a homodimer with subunit molecular weights between 67 kDa and 75 kDa.

2.4. Stability and optimum pH for activity

Native human holoTK is remarkably stable. Erythrocyte TK in crude extracts loses activity only when it is repeatedly frozen and thawed [150]; it remains active over two weeks while undergoing purification at 24°C [10]. When whole blood was stored in acid dextrose at 5°C, the erythrocyte TK activity was not lost after several days and at −20°C activity remained stable for up to a month [181]. Human TK purified from brain retained 80%–90% of its initial activity after storage at −20°C for one month, and recombinant human TK, expressed in *E.*

coli, remained stable in the crude extract even when stored at ambient temperatures for several weeks [137]. The stability of human TK is greatest at a pH between 7.5 and 10 [56]. The loss of activity after incubation of a pure preparation of this enzyme at 55°C for 5 min was 50%; however, when albumin was added prior to the incubation the loss was only 3% [158]. Heat-treatment of a crude extract at 55°C has been an early step in some purification protocols for human TK because it destroys the heat-labile TA, whereas the loss of TK is small [157]. Pig liver TK [120, 147] and rat liver TK [115] also display relatively high stability with a pH optimum between 7.8 and 8.2. In the presence of 4 mM ThDP incubation of the porcine enzyme for one hour at 50°C does not lead to any measurable decrease in activity [120]. Rabbit liver TK seems to be less stable than the other characterised mammalian TKs; when stored at −20°C in the presence of phenylmethylsulphonyl fluoride, mercaptoethanol and EDTA, 46% of its activity was irreversibly lost after 70 days [98].

The removal of ThDP from mammalian TKs requires acidic conditions, whereas resolution of the yeast [154], *C. utilis* [79] and *E. coli* [153] enzyme occurs at alkaline pH. Consistently, purified baker's yeast TK stored under alkaline conditions appears to be less stable than the mammalian ones. A loss of more than 30% of the activity after two weeks was reported when the enzyme was stored at −20°C in the presence of glycerol and dithiothreitol (DTT) at pH 7.9 [20]. However, when stored at −20°C as a crystalline suspension in saturated ammonium sulphate the enzyme remained stable for months [82]. The purified enzyme from *E. coli* in glycylglycine buffer at pH 8.5 could be stored at −20°C in the presence of DTT and 20% glycerol for three months with less than 20% loss of activity [153]. At 4°C in glycylglycine buffer at pH 8.5 the loss of activity was approximately 10%/month. When kept at a lower pH in phosphate buffer (pH 7.0) at −20°C *E. coli* TK in a cleared lysate retained 100% of its activity for a month [103].

The optimum pH for activity is similar in all characterised TKs. For the enzymes from human erythrocytes [56] and *C. utilis* [79] the optimal pH is 7.7. A value between pH 7.5 and 7.6 has been reported for TKs from baker's and brewer's yeast [24, 130], as well as from spinach leaves [165]. An optimal pH range between 7.4 and 8.2 has been observed for liver TK from various species [98, 115, 120, 147] and the *E. coli* enzyme displays maximum activity in glycylglycine buffer at pH 8.0 to 8.5 [153] and between pH 7.0 and 7.5 in phosphate buffer [103].

2.5. Cofactor requirements

ThDP can be removed from baker's yeast TK under relatively mild, alkaline conditions [24]. After complete resolution the addition of ThDP and a divalent cation are absolutely necessary to restore catalytic activity [57]. It has also been shown that a loss of activity can be prevented by the addition of cofactors during the purification of TKs from yeasts [20, 24, 57, 74, 124, 156], consistent with the pH conditions of resolution procedures being similar to the pH conditions of optimised purification protocols. In contrast, partially purified mammalian TKs do not require the addition of cofactors to maintain activity. This observation suggests a greater affinity of the mammalian enzymes for the cofactors [56, 81, 98], a proposition that is also reflected in the conditions required for the resolution of these enzymes [63, 72, 161], which is achieved at pH values below 4 [10, 56, 170], well removed from the pH conditions of purification procedures.

Purified holoTKs from yeasts and mammalian sources have been reported to contain one molecule of ThDP per subunit [46, 56, 72, 81, 98, 116, 120, 158], a result which has been confirmed, at least for the *S. cerevisiae* enzyme, by the crystal structure [93, 111].

The necessity of divalent metal ions for activity has been shown for baker's yeast TK [57, 124]. The reconstitution rate of this enzyme increases in the order $Ni^{2+} < Mg^{2+} < Co^{2+} < Mn^{2+} < Ca^{2+}$, but the final catalytic activity is independent of the nature of the divalent metal ion [57]. A similar observation has been reported for *E. coli* TK [153]. Initially, it was suggested that mammalian TKs do not require any divalent cat-

ions for activity [120, 172]. However, reconstitution studies in the presence of ethylendiaminetetraacetic acid (EDTA) have demonstrated that a divalent cation is absolutely necessary for activity [72]; activity may be restored by Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+}, but not Zn^{2+} or Cu^{2+}.

2.6. Sequence and evolutionary analysis

A detailed sequence comparison has shown that the overall sequence homology between TKs from divergent species is quite low [138]. Nonetheless, there are two regions with a high degree of sequence similarity. The first one, located in the N-terminal domain, is the motif common to all ThDP-dependent enzymes (Fig. 2) [52]. A second motif, which has an even higher degree of sequence homology, was originally discovered because of its similarity to the nucleotide binding motif of NADH-dependent dehydrogenases [1]. Subsequent analysis has shown that this motif (Fig. 5) is specific for TKs and it has therefore been denoted the "TK motif" [138].

A phylogenetic analysis of TK sequences has revealed that this enzyme reflects taxonomic relationships quite accurately. Its slow evolutionary dynamics together with its ubiquitous distribution may make this ancient enzyme a suitable model for a molecular clock, in particular for mammals [138].

3. Metabolic roles of TK

TK, together with TA, reversibly links glycolysis to the PPP. TK catalyses the conversion of D-xylulose 5-phosphate and D-ribose 5-phosphate to D-glyceraldehyde 3-phosphate and D-sedoheptulose 7-phosphate (Fig. 4). It is recognised that TK and TA together can form the products D-fructose 6-phosphate and D-erythrose 4-phosphate, a precursor of aromatic amino acids. Further, TK can catalyse the conversion of D-xylulose 5-phosphate and D-erythrose 4-phosphate to D-glyceraldehyde 3-phosphate and D-fructose 6-phosphate. TK transfers a two-carbon unit ("activated glycolaldehyde", intermediate 1 in Fig. 3), whereas TA, which does not contain a prosthetic group, transfers a three-carbon unit via the formation of a Schiff base. The carbohydrate that donates the two- or three-carbon unit is always a ketose and the acceptor is always an aldose. The reactions recognised as the PPP all occur in the cytosol. Thus, depending on the metabolic demand of the organism the PPP may provide precursors for biosynthesis or metabolites for glycolysis. However, the above reaction sequence of the non-oxidative branch of the PPP (Fig. 4) is based on an interpretation of the results of catalysis by liver and pea enzyme preparations incubated with variously labelled ^{14}C-ribose phosphate substrates and the resultant formation of ^{14}C-labelled fructose 6-phosphate. These reactions were tentatively attributed to catalysis by TK and TA [40, 64]. The conclusion concerning the nature and order of these reac-

Hsa	**SHCGVSIGEDGPSQMALE**DLAMF**R**SV**P**TSTVF**YP**S**D**
Cpl3	**THDSIGLGEDGPTHQPIE**HLASF**R**AM**P**NILML**RP**A**D**
Sce	**THDSIGVGEDGPTHQPIE**TLAHF**R**SL**P**NIQVW**RP**A**D**
Eco	**THDSIGLGEDGPTHQAVE**QLASL**R**LT**P**NFSTW**RP**C**D**
Consensus[a]	**SHDGVGIGEDGPSQMALE**xxxxx**R**xx**P**xxxxx**YP**x**D**
	T CSISL GN THQPID R D
	V V

[a]x denotes any amino acid

Fig. 5. TK motif. The sequences are taken from the multiple sequence alignment in Schenk et al. [138]. The sequences selected for illustration are from the same organisms as in Fig. 2 and the consensus sequence based on the entire alignment in Schenk et al. [138] is shown.

tions (Fig. 4) is still controversial because the degree of ^{14}C isotope labelling and its distribution in carbon atoms 1 and 3 of fructose 6-phosphate differed from that predicted by the reaction sequences shown in Fig. 4 [40, 64, 180]. One reason for those discrepancies might be that TK appears to catalyse exchange reactions much faster than the non-oxidative PPP flux rates [35].

Datta and Racker [24] reported that the equilibrium constant for reactions catalysed by TK is close to unity, indicating that the relative concentrations of substrates and products control the direction of the reaction. This result is consistent with a requirement for metabolic flexibility. It has been suggested for *S. cerevisiae* that TK and TA control the flux of metabolites through the non-oxidative limb of the PPP [133, 144]. NMR studies on human hemolysates have demonstrated that TK has a high flux-control coefficient, indicating that this enzyme exhibits a controlling function on the PPP in erythrocytes [5]. Although this pathway is ubiquitous there is evidence that its metabolic activity in higher organisms is tissue-dependent, e.g. its activity is low in skeletal muscle and liver, but very high in adipose tissue, mammary glands and the adrenal cortex, where large amounts of NADPH (generated in the oxidative branch of the PPP) are required for the reductive synthesis of fatty acids and steroids from acetyl CoA. The PPP is also active in human erythrocytes where the NADPH is required to maintain reduction of glutathione for the detoxification of reactive oxygen species.

Several studies have shown that PPP activity is greatly increased in rabbit, rat and mouse mature corneal epithelia [49, 88, 131]; in bovine corneal epithelium 35% of glucose is metabolised via the PPP [78]. It is very likely that this high activity reflects the need of the corneal tissue for sufficient amounts of reducing equivalents (NADPH) for the prevention of damage by H_2O_2 and free radicals formed by UV radiation [49, 131]. In newborn mice a great increase in TK synthesis was observed at the time of the eye opening [131]. In the corneal epithelium of mouse 10% of the soluble protein is TK [131]. While TK is the rate-limiting enzyme in the nonoxidative link of the PPP (see above), it is yet unknown if other enzymes of the PPP are also expressed in such great abundance in these tissues. The high concentration of TK in ocular tissues suggests the proposition that this enzyme might also be structurally important for light refraction, tissue transparency and light absorption, i.e. TK might be an enzyme-crystallin of the lens, similar to other putative enzyme-crystallins such as glutathione S-transferase [131].

Although data about plant TKs are scarce it is clear that this enzyme forms an important link between the Calvin cycle and carbohydrate metabolism (e.g. PPP and glycolysis) on the one hand and on the other hand anabolic pathways leading to the formation of nucleic acids, amino acids and many derivatives of these. In the desiccation-tolerant plant *C. plantagineum* one TK gene is constitutively expressed. Two additional ones, however, are synthesized only during the process of rehydration [4] perhaps in order to increase the flux of carbohydrates through the PPP to satisfy the increased demand for nucleotides and amino acids during the accelerated growth of rehydrating plants.

A mainly anabolic role can also be inferred for the recP gene product from *Streptococcus pneumoniae*. A sequence comparison reveals that the recP gene, whose product is involved in the recombination process [126], is likely to encode a TK [138, 155]. It is postulated that this enzyme is required during recombination for the synthesis of abundant quantities of ribose 5-phosphate for the subsequent generation of phosphoribosyl pyrophosphate, a precursor in the biosynthesis of nucleotides.

4. Catalytic mechanism of TK

TK catalyses the interconversion of monosaccharides by transferring glycolaldehyde moieties from ketose donors to aldose acceptors (Fig. 4). The catalytic mechanism is depicted in Fig. 3. The crystal structure of *S. cerevisiae* TK reveals that the cofactor Mg^{2+}-ThDP complex in this enzyme is bound in a hydrophobic pocket at the interface of the two subunits and only C2 of the

thiazolium ring is accessible from the solvent [93, 101, 111]. The abstraction of the proton from C2 is the initial step in catalysis in all studied ThDP-dependent enzymes. For TK the following model has been proposed for the mechanism that leads to the formation of the carbanion (ThDP ylide in Fig. 3) [142].

The invariant residue Glu418 (unless otherwise stated the numbers refer to the *S. cerevisiae* sequence [155]) is sufficiently close to form a hydrogen bond with N1′ of the aminopyrimidine ring. The importance of this interaction has been demonstrated by experiments in which the putative hydrogen bond was disrupted either by deaza analogues of the cofactor [45, 84] or by site-directed mutagenesis of residue 418 [175]. It is proposed that this hydrogen bond promotes the generation of a resonance form with a positively charged imino-group at the 4′-position of the pyrimidine ring. The side chain of His481 is close to this charged imino group and it has been suggested that His481 (which corresponds to His113 or His114 in *Zymomonas mobilis* PDC which have been shown to be involved in various aspects of PDC function [19, 139]) may abstract the proton from the imino group, which would increase the pK_a of the imino group sufficiently that it could in turn remove the proton from the C2 of the thiazolium ring. One problem of this mechanism is that in mammalian TKs His481 is replaced by a glutamine [138], rendering the proposed catalytic function of His481 questionable. Singleton and his colleagues [149] have made a mutant human TK in which this residue (Gln428 in mammalian TKs) has been replaced by a histidine (the residue in all nonmammalian TKs in this position). It displays only 30% of the wild-type activity, consistent with the proposition that the functions of these corresponding residues in mammalian and nonmammalian TKs are not identical. The authors have postulated that this glutamine might be involved in stabilising and orienting a water molecule required for catalysis [149]. However, without the crystal structure of a mammalian TK the effects of the observed differences at this residue remain speculative.

After formation of the ThDP ylide, binding of the donor substrate and a nucleophilic attack on the carbonyl carbon atom of the substrate are the next steps in catalysis. The three-dimensional crystallographic data of *S. cerevisiae* TK clearly indicate a substrate channel [111, 112]. It has been suggested that an interaction between the C1-hydroxyl group of the substrate and the side chain of the invariant residue His103 may be required for optimal positioning of the donor substrate for the nucleophilic attack [176]. The formation of an adduct between the donor substrate and cofactor (intermediate 1 in Fig. 3) is accompanied by the protonation of the carbonyl oxygen of the substrate. Again, His481 has been proposed as the most likely candidate for the proton donor according to the structure of *S. cerevisiae* TK [142]. In a recent study carried out by Schneider's group His481 was replaced by alanine, serine and glutamine [177]. The three mutants had a drastic decrease in catalytic activity and the affinity for the donor substrate was strongly reduced. These results are consistent with the proposition that His481 is likely to be important for the stabilisation of intermediate 1 (Fig. 3), at least in yeast TK. Subsequently, the abstraction of the proton from the C3-hydroxyl group of the substrate, possibly mediated by a concerted action of the invariant residues His30 and His263 [177], leads to the cleavage of the addition compound and the release of the first product, an aldose sugar. Concomitantly, the 2-α-carbanion of the reaction intermediate α,β-dihydroxyethyl-ThDP (DHEThDP, also named the 'active glycolaldehyde') is formed. Recently, it has been shown that DHEThDP can act as the donor substrate for TK from baker's yeast with a reaction rate that is much the same as that of natural substrates [146, 163]. These results suggest that the deprotonation that leads to the formation of the 2-α-carbanion is not a rate-limiting step in TK catalysis.

The generation of this 2-α-carbanion is common to all reactions catalysed by ThDP-dependent enzymes. In TK this carbanion reacts with an aldose acceptor substrate to form intermediate 2 (Fig. 3). The crystal structure of *S. cerevisiae* TK with bound acceptor substrate has indicated

which amino acid residues of the substrate channel may interact with the substrate [112]. However, these structural data do not necessarily reflect the actual geometry during catalysis because this enzyme–substrate complex is lacking the glycolaldehyde moiety attached to ThDP (DHEThDP, see above). The glycolaldehyde moiety is transferred to the acceptor to form a new ketose sugar and simultaneously regenerate ThDP. This second part of catalysis is essentially a reversal of the first part and it is therefore likely that the same side chains will be involved in the proton transfer. The release of the first product, followed by the binding of the second substrate implies that the active site is accessible from the solvent during this exchange. It is not yet understood how the enzyme can stabilise the negatively charged reaction intermediate in this "open" conformation.

The majority of the evidence for the catalytic mechanism of TK has been obtained from structural studies of the yeast enzyme [e.g. 142]. Kinetic studies of DHAS from *C. boidinii* [74] and TK from *E. coli* [50] have shown that the properties of these enzymes are consistent with this "ping-pong" mechanism [22]. Most of the kinetic analyses have been focussed on the investigation of the interactions between TK and its substrates [e.g. 143] and the binding of ThDP to the enzyme [e.g. 11, 32, 161]. Yeast TK displays marked hysteresis [110] in binding ThDP [32] as does human TK [10, 148, 161]. In the case of the human enzyme, binding is so slow at the concentration of ThDP in erythrocytes (200 nM in health [159] and as low as 40 nM in thiamin deficiency [160]) that the measurement of an apparent K_m value for the binding of Mg^{2+}-ThDP to apoTK is technically demanding [161]. After one hour of incubation the apparent K_m value can be measured at 65 ± 14 nM [161] and after a three-hour incubation the value decreases further, to 15 ± 9 nM [137, 140], making the binding of ThDP to TK tight as well as slow.

5. Industrial uses of TK

It has been proposed that an increase in the PPP activity would increase the fermentation efficiency of *S. cerevisiae* for particular industrial applications. However, all attempts to produce large quantities of ethanol by means of recombinant yeasts overexpressing TK and TA have failed thus far [e.g. 96, 168]. Nonetheless, TK finds an increasing number of applications for industrial purposes, in particular in the synthesis of chemicals. One example is the biosynthesis of the aromatic amino acids L-phenylalanine, L-tryptophan and L-tyrosine from D-glucose [e.g. 39]. These amino acids are used as precursors for the organic synthesis of various products; e.g. L-phenylalanine can be transformed into the artificial sweetener aspartame [166], L-tryptophan into the dye indigo [34] and L-tyrosine into eumelanin, a UV-absorbing substance [26]. Attempts to increase the conversion of D-glucose into aromatic amino acids originally focussed on the enzyme 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthase [121], the first enzyme unique to the common pathway of aromatic amino acid biosynthesis [174]. DAHP synthase catalyses the irreversible condensation of phosphoenolpyruvate with D-erythrose 4-phosphate resulting in the formation of DAHP and inorganic phosphate [121]. However, the supply of D-erythrose 4-phosphate requires the reactions of the PPP, particularly those catalysed by TK, and in an *E. coli* strain overexpressing TK the proportion of D-glucose directed towards synthesis of aromatic amino acids is significantly increased [29, 47].

TK is also an important biocatalyst in stereospecific carbon–carbon bond synthesis [60]. TK accepts a large number of aldehydes as substrates [59, 67, 106], in particular those containing an α-hydroxy group in 2R configuration; the products are ketoses with a 3 S,4R configuration (threo configuration) [27, 31]. As an example, TK from spinach can catalyse a reaction between 2,3-dihydroxybutyraldehyde and hydroxypyruvate, yielding a mixture of 6-deoxy-D-fructose and 6-deoxy-L-sorbose [53] which can serve as precursors of 2,5-dimethyl-4-hydroxy-

3(2H)-furanone (furaneol), an important industrial aromatic product with caramel-like flavour used in the food industry [54]. One of the major problems for the large scale industrial use of TK-catalysed biotransformations is that high concentrations of substrate strongly decrease the turnover number [9, 103, 137]. Using hydroxypyruvate and glycolaldehyde as substrates Mitra et al. [103] analysed limitations for an optimal turnover of product (see above). At high concentrations (500 mM) of the acceptor substrate (glycolaldehyde) TK was inactivated. However, development of an enzyme-membrane reactor which keeps the concentrations of substrates and products low greatly increases the half-life of TK and the yield of reaction products [9].

6. Variant forms of TK

The first report of apparent multiple forms of TK was published almost 30 years ago by Kiely et al. [77]. These authors showed that the enzyme, purified from *C. utilis*, eluted in three peaks from a DEAE-Sephadex column. However, another group were able to find only one peak of *C. utilis* TK activity eluting from the ion exchange column [79]. Another example of multiple forms was found during chromatography of human erythrocyte TK on a Mono P column, which separates the enzyme activity into three peaks [158] but amino acid analysis and immunological studies indicated that the three peaks appeared to be the same protein. Based on their separation on phosphocellulose and thermostability, multiple forms of TK have also been reported for this enzyme purified from baker's yeast, the livers of rat and pig and several organs of rabbit [89]. It was also shown that the different forms are not an artefact of the purification procedures. The same group later reported different morphologies for the crystals of the multiple TK forms [90]; these TK variants can interconvert, indicating that they are not different isozymes. Kuimov et al. [90] speculated that posttranslational modifications or a reversible association with a ligand may be responsible for the existence of multiple TK forms but as yet no solid evidence in support of this proposal has been published.

However, it has become evident that some organisms do possess true variant forms of TK. In 1993 two groups independently cloned two *S. cerevisiae* TK genes which have approximately 70% identity [132, 155]. This was the first convincing evidence for genetic heterogeneity of any TK. Since then two genetic variants have been found in the bacterium *E. coli* [68, 152] and three in the plant *C. plantagineum* [4]. While it is still unclear why *E. coli* and *S. cerevisiae* have two different TKs it could be shown that only one of the three TK forms in *C. plantagineum* is expressed constitutively. The remaining two forms appear to be involved specifically in the rehydration process [4].

The occurrence of variant forms of human TK has been controversial since it was first reported by Blass and Gibson in 1977 [7]. They measured the affinity of apoTK for its cofactor ThDP, using unpurified enzyme from cultured human fibroblasts. Their results showed that the enzyme isolated from healthy controls had a higher affinity for the cofactor than the enzyme extracted from fibroblasts of patients suffering from the neurodegenerative disorder Wernicke–Korsakoff (WK) syndrome. The authors suggested that the patients were genetically predisposed to this disorder. Their results were later supported by Mukherjee et al. [107] although the observed difference between the apparent cofactor binding constants for patients and controls was much smaller. In this laboratory TK isolated from erythrocytes of WK syndrome patients and controls did not display any variation in ThDP affinity [113]. It was pointed out that variant apparent K_m values may be due to variations in the applied assay conditions given the tight, slow binding of apoTK with the Mg^{2+}-ThDP complex [161].

Studies applying isoelectric focussing did not satisfactorily resolve the controversy about a putative polymorphism of human TK. When a pure erythrocyte TK preparation from a single individual was analysed by application of this method, the enzyme resolved into three to eight distinct bands [73]. Focussed bands containing

TK were detected by a specific activity stain [182]. Multiple bands of isoelectrically focussed TK have also been found in human leukocytes [105] and rat liver [116]. A comparison of the banding patterns of focussed TK isolated from red blood cells of WK syndrome patients and healthy controls led to the report that one particular banding pattern apparently correlates with this disorder [113]. However, Kaufmann et al. [75] reexamined the pattern of erythrocyte TK from 63 healthy subjects. While seven bands could be readily reproduced from all samples four additional bands occurred in some but not in other samples. The fluctuation in the banding pattern of healthy subjects led to the conclusion that the absence or presence of particular bands was not a sufficient proof for the occurrence of genetic variants of human TK. Blansjaar et al. [6] compared isoenzyme patterns of WK patients, their relatives, alcoholics and healthy subjects and they found no evidence for a genetic variation. More importantly, the cDNA encoding TK has been sequenced from two healthy controls and two patients suffering the WK syndrome and no consistent non-silent mutations distinguished these two groups [100]. However, none of these studies has ruled out the possibility that differences in postranslational modifications could be responsible for observed variant forms of TK. For example, purified rat liver TK has been phosphorylated in vitro by protein kinase C and to a smaller extent by cAMP-dependent protein kinase and casein kinase II [151] but evidence of phosphorylation of TK in vivo is yet to be published. The results of such experiments are eagerly awaited since in vitro phosphorylation decreased TK activity [151].

While the possibility of genetic variations of human TK has not been ruled out, it seems unlikely that multiple genes encode this enzyme. Using as a probe DNA containing part of the human TK gene sequence (cloned from a human liver genomic DNA library [1]), the location of the TK gene has been mapped; only one locus hybridised to the probe [92]. Unless there is a distinctly different form of TK (which would not hybridise with the chosen genomic DNA probe) it is very likely that there is only one gene encoding human TK. However, the recent cloning of transcripts of human TK-related genes from different tissues has raised the possibilities of alternative splicing and formation of heterodimers [23]. A comparison with the sequences of known TKs showed that the TK-related genes contain the ThDP-binding (Fig. 2) and TK (Fig. 5) motifs. However, since several important amino acid residues are missing from the translated TK-related sequences these putative gene products may be nonfunctional. Additional sequence variations were discovered between TK-related genes from different tissues (heart and brain). Coy et al. [23] hypothesise that different forms of TK activity could be due to allelic variations of the TK-related gene, but so far no expression product of this gene has been reported.

7. Potential associations of TK activity with disorders consequent to thiamin-deficiency

Like other ThDP-dependent enzymes, the activity of TK is decreased in conditions of thiamin deficiency. The best known thiamin deficiency disease, beriberi, has been little studied as to its enzymologic consequences since the most consistent pathology is in fine peripheral nerves which make poor samples from which to isolate enzymes. However, the WK syndrome causes characteristic, localised brain pathology and the consequences of thiamin deficiency for the brain have been well studied. Of all the ThDP-dependent enzymes in the brain, TK activity decreases first and returns last following thiamin repletion of a thiamin-deficient animal [44, 99, 164]. However, it appears that these changes in TK activity do not directly lead to the localised neuropathology because the changes in activity correlate poorly with changes in the manifestation of disease [44]. Moreover TK protein and TK mRNA are distributed unequally amongst rat brain nuclei, but their decreases in the thiamin deficient rat brain do not correlate with the sites of brain pathology [145]. In fact, α-ketoglutarate dehydrogenase (KGDH) is the ThDP-dependent enzyme whose activity varies, in thia-

min deficiency, in the closest temporal relationship with the manifestations of the disease in experimental rats [16, 17, 43].

Erythrocyte TK activity is measured to assess human thiamin deficiency and assays of hemolysate TK activity in the absence and presence of cofactor added in vitro allow the inference that some apoTK accumulates as holoTK decreases in human thiamin deficiency [76, 114, 123]. Indeed the rapid symptomatic response of thiamin-deficient patients to therapeutic thiamin is paralled by the apparent conversion of erythrocyte apoTK to holoTK in vivo within a period as brief as 30 min [123]. Moreover, the total erythrocyte TK activity is restored to the thiamin-replete levels, consistent with the retention of total TK protein throughout the period of thiamin deficiency. However, in hemolysates from thiamin-deficient patients, not all the apoTK can be reactivated in vitro, for reasons that are as yet unclear [41, 123]. Since mature erythrocytes do not synthesise protein, the half-life of erythrocyte TK protein must be similar to or greater than the half-life of the erythrocyte, i.e. 50 days in humans. The half-life of TK protein in other tissues might be quite different from that of erythrocytes. It has been measured in rat heart, where it is 68 hours [87] and in cultured human lymphoblasts, where it was only 1.4 hours, whether the culture medium was replete or deficient in thiamin [119]. However, thiamin deficiency appeared to decrease the rate of TK protein synthesis in these cultured lymphoblasts [119]. Such studies need to be repeated in whole animals and other cultured cells to determine whether the results are peculiar to lymphoblasts.

8. TK changes in Alzheimer's disease patients

In brains and other tissues from Alzheimer's disease patients examined post mortem, there is decreased activity of TK [42]. Moreover, isoelectric focussing has detected a change in TK extracted from fibroblasts grown from Alzheimer's patients [117]. This change could be attributed to proteolytic cleavage of TK resulting in a shorter subunit that retained some activity. Subsequently, it has been shown that a cysteine proteinase in the fibroblasts of Alzheimer's patients is responsible for this activity [118]. Thus, these changes in TK activity appear to represent a marker for Alzheimer's disease, but there is not yet any evidence that they are causally related to the pathogenesis of the disease.

9. Conclusions and future directions

Recent analyses of the catalytic mechanism of TK have derived an increasingly complex model which has been partly explained by the structure of the *S. cerevisiae* TK at 2.0 Å resolution. However, the structure has posed new questions. It has been observed that the two active sites in TK display non-equivalence although, according to the crystal structure, they appear to be identical. Neither the underlying cause nor the significance for the occurrence of this phenomenon is well understood; introduction of specific mutations into TK and/or crystallographic resolution of a binary complex of TK with a bound donor substrate analogue will be necessary to maintain the proposition that this apparent non-equivalence of the two active sites depends upon an alternating direction of electron flow from one ThDP to the other ThDP via hydrogen bonds which link these catalytic centres. Elucidation of the structure of a mammalian TK is eagerly awaited. This will not only shed light on mechanistic properties of this enzyme but also reveal insight into functional variations observed between mammalian and nonmammalian TKs, e.g. substrate specificity and cofactor binding.

TK actvity is crucial for the flux of carbohydrates through the non-oxidative limb of the PPP and understanding its control is critical to the industrial application of TK in the production of biosynthetic molecules. Recombinant mutants possessing desired features such as broader substrate range, higher thermostability or increased turnover rate may be designed for use in industrial processes. Generation of recombinant microbial systems expressing TK has commenced and some encouraging results have been reported. Recent analyses have outlined the

requirements for a bioreactor carrying out TK-catalysed reactions; further optimisation for large-scale operation at high concentrations of reactants would be facilitated by engineering TK to enhance the affinity for substrates such as hydroxypyruvate and glycolaldehyde, while increasing the turnover rate and decreasing the potential for substrate inhibition by hydroxypyruvate.

The role of TK in the PPP has been well recognised for more than 40 years. Interestingly, recent research has indicated that TK may have not only a metabolic role but also a structural one, at least in the cornea. It is not yet known if the large abundance of TK in these cells is due to the proposed structural function, or if it is simply a reflection of the eye's requirement for a high PPP activity in order to maintain the necessary reducing environment. Measurements of the levels of other enzymes of the PPP, in particular that of glucose 6-phosphate dehydrogenase (the rate-limiting enzyme of the oxidative branch) would help to clarify this issue.

It has become increasingly clear that during thiamin deficiency in humans the changes of TK activity do not reflect the actual course of the disorder. Furthermore, the search for human TK genetic variations associated with thiamin deficiency has failed. Thus, unless it can be shown in vivo that tissue or individual specific variations or posttranslational modifications cause changes in TK activity, there is no evidence for a direct involvement of TK in the pathogenesis of disease. One further possible source of human TK variants is the finding of alternatively spliced transcripts of a TK-related gene, but it is yet to be shown that these transcripts are translated and that the product has TK activity. However, partial proteolytic cleavage of TK does serve as a marker for Alzheimer's disease and hence TK will remain an interesting candidate for neurobiochemical studies.

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