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# Heterologous expression of human transketolase

Gerhard Schenk, Ronald G. Duggleby, Peter F. Nixon *

*Centre for Protein Structure, Function and Engineering, Department of Biochemistry, The University of Queensland, St. Lucia, QLD 4072, Australia*

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**Abstract**

Transketolase belongs to the family of thiamin diphosphate dependent enzymes. The aim of this study was to establish a bacterial expression system for human transketolase in order to investigate the functional characteristics of mammalian transketolases. The level of recombinant human enzyme expressed in *Escherichia coli* was modest. Purification of recombinant transketolase and separation from the *E. coli* enzyme has been greatly simplified by means of a non-cleavable hexa-histidine tag. The highest specific activity was 13.5 U/mg and the $K_m$ values were $0.27 \pm 0.02$ and $0.51 \pm 0.05$ mM for the substrates D-xylulose 5-phosphate and D-ribose 5-phosphate, respectively. Binding of cofactors to the apoenzyme showed the expected hysteresis. Without preincubation, the $K_m$ values for thiamin diphosphate and for $Mg^{2+}$ were, respectively, $4.1 \pm 0.8$ and $2.5 \pm 0.4$ $\mu$M, but after 1 h of preincubation these values were $85 \pm 16$ nM and $0.74 \pm 0.23$ $\mu$M. The kinetic constants are similar to those of the native enzyme purified from human erythrocytes. Despite the modest expression level the reported system is well suited to a variety of functional studies. 

*Keywords:* Thiamin diphosphate; Heterologous expression; Protein purification; Kinetic parameters; Recombinant human transketolase

## 1. Introduction

Transketolase (TK), a homodimeric, ubiquitous enzyme of energy metabolism, requires thiamin diphosphate (ThDP) and a divalent metal ion (e.g. $Mg^{2+}$) as essential cofactors and catalyses the transfer of glycoaldehyde moieties from ketoses to aldoses in the pentose phosphate pathway (PPP). This pathway requires great flexibility in order to respond appropriately to metabolic demands. Depending on the state of the cell the PPP can provide precursors for the biosynthesis of nucleotides and amino acids, or donate metabolites for glycolysis. Together with transaldolase, TK catalyzes the interconversion of those compounds; it has been suggested for *Saccharomyces cerevisiae* that TK and transaldolase control the flux of metabolites through the PPP [21, 24] and NMR studies on human hemolysates demonstrated that TK has an high flux-control coefficient, indicating that this enzyme indeed exhibits

* Corresponding author. Tel.: +61-7-33654613; fax: +61-7-33654699; e-mail: nixonp@biosci.uq.edu.au.

Abbreviations: IDA, Iminodiacetic acid, PPP, Pentose phosphate pathway, R 5-P, D-Ribose 5-phosphate, ThDP, Thiamin diphosphate, TK, Transketolase, WK, Wernicke–Korsakoff, X 5-P, Xylulose 5-phosphate.

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a controlling function on the non-oxidative limb of the PPP [2].

Due to its central role in energy metabolism TK has been under investigation for many years. The enzyme has been isolated from various organisms, but only a few TKs, namely those from *Escherichia coli* [28], *S. cerevisiae* [9, 12, 15, 18, 33, 34] and human [4, 5, 13, 30, 32], have been characterised well. A comparison between these three homologous enzymes has revealed that physicochemical properties such as the pH optimum or the stability of the activity are very similar. Previously, the three-dimensional structure of *S. cerevisiae* TK has been resolved to 2.0 Å [18] and preliminary crystallographic data on the *E. coli* enzyme have been published [16]. This structural information allowed the identification of residues predicted to be involved in various parts of the enzyme's function, e.g. catalysis, subunit interaction, substrate and cofactor binding. A detailed protein sequence comparison between most known TKs has shown that particular residues are conserved [23]. However, while bacterial, fungal and plant sequences could readily be aligned, major gaps had to be introduced into the mammalian sequences to allow alignment [23]. This result is consistent with the smaller subunit molecular weight of mammalian TKs. Also, an entire α-helix (α-11) appears to be absent in mammalian TKs which certainly would have an effect on these enzymes' overall structure. Consistent with those findings it was observed that mammalian and nonmammalian TKs display distinct differences in interactions between the subunits, in cofactor binding and in their hysteretic behaviour. Additionally, the mammalian TKs have a much smaller range of utilizable substrates than the other TKs. Clearly there is evidence for functional and structural variations between the two groups. An examination of the subtle functional variations between bacterial, fungal and human TKs may lead to a more detailed analysis of the catalytic mechanism of these three TKs and ultimately to a more comprehensive understanding of ThDP-dependent enzyme catalysis in general. Furthermore, the elucidation of the three-dimensional structure of the human enzyme would allow comparison of the structures of the three enzymes.

The availability of a recombinant expression system for a protein of interest provides a tool for its rapid and simple production, its easy manipulation and facilitates studies on the structure and function of the protein. Homologous expression systems for TK from *E. coli* and *S. cerevisiae* have been reported previously [28, 29]. Here we describe an heterologous expression system for the human enzyme.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*E. coli* strain DH5α was obtained from Gibco BRL. Plasmid pTrc99A was purchased from Pharmacia and the heat-inducible expression vector pCL476, containing the sequence coding for a non-cleavable hexa-histidine tag adjacent to the multiple cloning site, was a gift from Dr. N.E. Dixon, Research School of Chemistry, Australian National University. All *E. coli* cultures containing the recombinant constructs were maintained on LB plates [19] with 100 μg/ml ampicillin. For long term storage liquid cultures were kept in 15% glycerol and stored at −20°C.

### 2.2. DNA manipulation

Ligations, transformations, restriction digests, preparation of plasmid DNA and agarose gel electrophoresis were carried out according to standard protocols [19]. Automated sequencing, applying the dideoxy chain termination method [20], was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit according to the manufacturer's instructions. PCR and sequencing reactions were carried out on a Perkin Elmer Cetus model 480 PCR machine.

### 2.3. Construction of pCL476/TK

The cloning of the 3′-end of human transketolase by our laboratory has been reported pre-

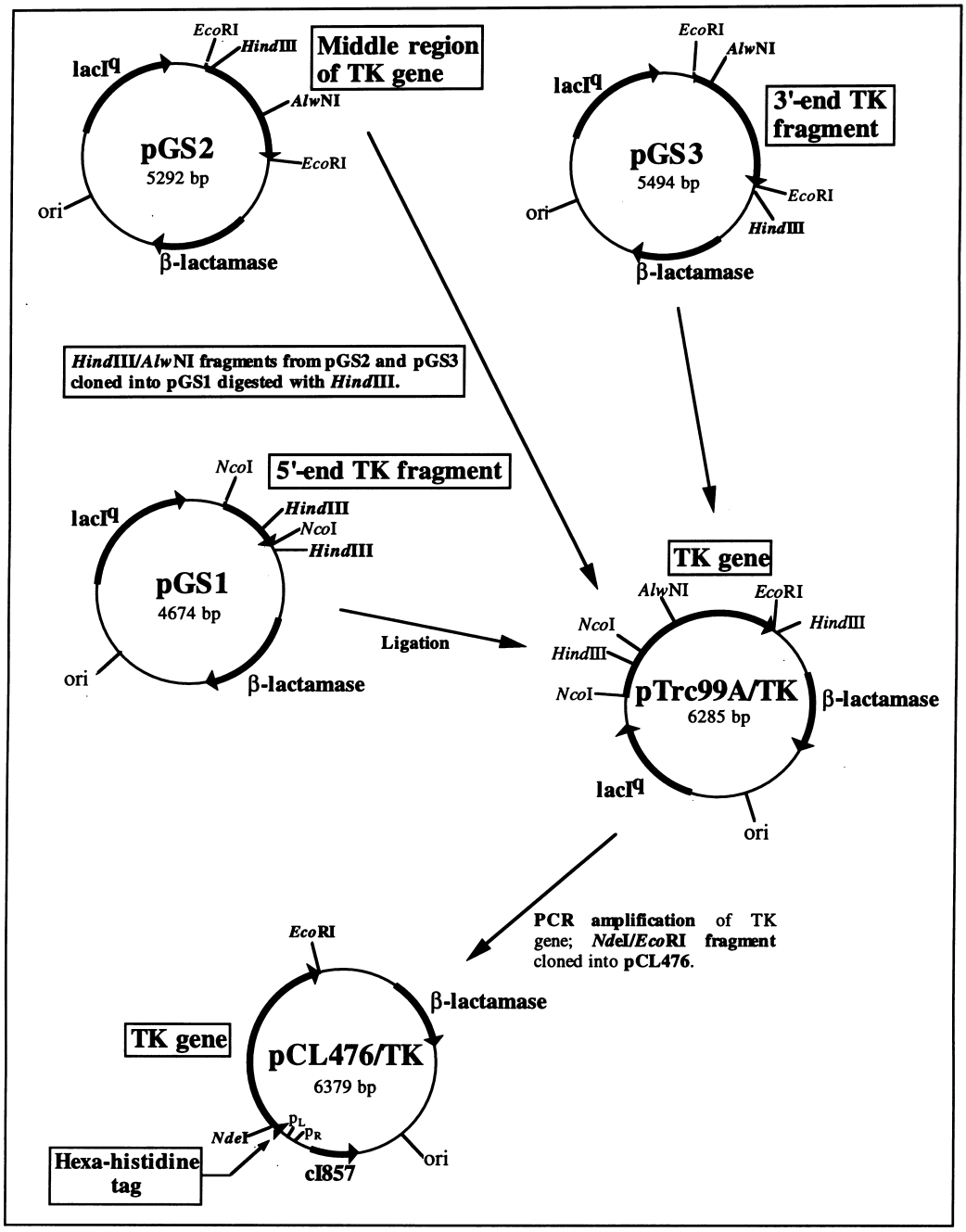


Fig. 1. Cloning strategy for human TK. The *Alw*NI/*Hind*III fragments of pGS2 and pGS3 were simultaneously cloned into pGS1 digested with *Hind*III, generating clone pTrc99A/TK which contains the complete DNA sequence of recombinant TK. PCR amplification of TK introduced an *Nde*I site at the 5′-end; the *Nde*I/*Eco*RI fragment was cloned into the multiple cloning site of pCL476.

viously (clone pTK2) [1]. Subsequently, pTrc99A/TK and pCL476/TK were constructed as follows (Fig. 1). First, an *Eco*RI fragment of pTK2 was inserted into the polylinker of pTrc99A, producing pGS3. Next, total RNA from human brain tissue was extracted using the GlassMAX RNA Microisolation Spin Cartridge System from Gibco BRL. Respective fragments containing nucleotides 1 to 498 (5′-end) and 303 to 1419 (middle region) of the coding region of TK were amplified by RT-PCR, using the GeneAmp RNA PCR kit from Perkin Elmer. The design of the oligo nucleotides, used for the amplification of the RT-PCR fragments, was based on the previously published human TK sequence [1, 17]. The following primers were used: (a) 5′-end 5′-TGC CGC ACC ATG GAG AGC TAC CAC-3′ (forward) and 5′-TTG TCC CCA AGC TTG TAG CTG GG-3′ (reverse) and (b) middle region 5′-TGA ACC TGA GGA AGA TCA GCT CCG-3′ (forward) and 5′-CAT AGC CAG ATC TTA AGG GGC CAT CTG GG-3′ (reverse). These fragments were cloned into pTrc99A, producing pGS1 and pGS2, respectively. pGS2 and pGS3 were digested with *Alw*NI and *Hind*III and the *Alw*NI/*Hind*III fragments from these two constructs were simultaneously cloned into pGS1 digested with *Hind*III. The resulting construct (pTrc99A/TK) contains the entire coding region for TK, which was amplified by PCR and cloned into the *Nde*I and *Eco*RI site of pCL476, producing pCL476/TK.

### 2.4. Sequence analysis and comparison

The complete nucleotide and deduced amino acid sequences were compared with those of fetal human TK [14] and the previously deposited sequence of human TK published by McCool et al. [17]. The sequences were aligned unambiguously by use of Clustal W software [31] and no further refinement was necessary.

### 2.5. Expression and protein purification

For large scale expression of TK the cells were grown in 10 l of 2YT medium [19] (containing 100 μg/ml ampicillin) at 30°C in a 20 l Chemap fermenter with aeration and stirring (320 rpm). When the cell culture reached an $OD_{600}$ of 0.5 the temperature was rapidly increased to 42°C. The induction was maintained for 3 h. The cells were harvested by centrifugation at 4°C for 16 min at 2600*g*. The cell pellet was stored at −20°C.

Recombinant TK was purified as follows. The cells were resuspended in lysis buffer (6 ml lysis buffer per g cell paste), containing 100 mM $KH_2PO_4$ (adjusted to pH 6.8 with KOH), 0.1 mM ThDP, 1 mM $MgCl_2$, 14.2 mM β-mercaptoethanol, 0.6 mg/ml lysozyme, 0.01 μg/ml DNaseI, 20 μg/ml leupeptin, 0.02 U/ml aprotinin, 0.3 μg/ml bestatin and 100 μM phenylmethylsulphonyl fluoride. The cells were disrupted using a French press (40 K Rapid-Fill Cell; SLM AMINCO Instruments). The lysate was purified by means of a one-step hydroxylapatite batch procedure (1 g hydroxylapatite (Riedel-deHaen) per 6 ml lysate) [4, 7]. The protein was eluted with 20% ammonium sulphate and precipitated by addition of ammonium sulphate to 90% saturation. The mixture was stirred vigorously and incubated overnight at 4°C. The precipitate was collected by centrifugation at 4°C and 17000*g* for 20 min and resuspended in a minimal volume of binding buffer, containing 20 mM Tris (adjusted to pH 7.9 with HCl), 5 mM imidazole, 500 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM ThDP. The protein mixture was desalted by dialysis at 4°C for 16 h against three changes of binding buffer. TK was further purified by affinity chromatography on a nickel–iminodiacetic acid (IDA)–Sepharose 6BFF column (5 ml, Novagen), equilibrated with binding buffer. The enzyme was eluted by a linear imidazole gradient from 5 mM to 1 M. Peak fractions were pooled and stored at 4°C.

### 2.6. Preparation of apoenzyme

All glassware, plastic cuvettes and tips were soaked in 20% (v/v) $HNO_3$ and rinsed several times with metal-free water (18.2 mΩ/cm, MilliQ water purification system, Millipore). Reagents used for the preparation of apotransketolase and activity assays were passed through a Chelex 100

(BioRad) column to remove divalent metal ions. The removal of cofactors from human TK has been described previously [5].

### 2.7. Analytical methods

TK activity was measured by a coupled enzyme assay at 37°C based on the method of Smeets et al. [27] as modified by Booth and Nixon [5]. In general, unless otherwise stated, reactions were started by addition of TK. The amount of enzyme was chosen so that a steady decrease of NADH absorption could be monitored over 10 to 15 min. The activity unit is defined as the amount of enzyme that catalyses the formation of 1 $\mu$mol of glyceraldehyde 3-phosphate per min.

ThDP stock solutions were assayed spectrophotometrically; the $\varepsilon_{277}$ is 8520 $M^{-1}$ $cm^{-1}$. The $Mg^{2+}$ concentration in stock solutions was measured by atomic absorption spectrophotometry at 285.5 nm. Protein concentrations were measured by means of the bicinchoninic acid protein assay kit (Sigma Chemical Company) according to the manufacturer's instructions.

### 2.8. Substrate and cofactor binding studies

Michaelis constants for the donor substrate D-xylulose 5-phosphate (X 5-P) and acceptor substrate D-ribose 5-phosphate (R 5-P) were determined from the dependence of the enzyme reaction velocity upon substrate concentration. Substrate concentrations in each assay ranged from 10 $\mu$M to 50 mM for R 5-P (in the presence of a saturating concentration, 2 mM, of X 5-P) and 1 $\mu$M to 5 mM for X 5-P (10 mM R 5-P). Reactions were started by the addition of approximately 8 mU of holoTK.

The cofactor affinity of recombinant TK was measured from the dependence of the activity of the reconstituted holoenzyme upon cofactor concentration. The assay mixtures contained saturating concentrations of one cofactor and varying concentrations of the other. Cofactor concentrations in each assay ranged from 0.1 $\mu$M to 5 mM for $Mg^{2+}$ (1 mM ThDP) and 1 nM to 1 mM for ThDP (1.2 mM $Mg^{2+}$). The concentrations of the remaining reagents were as described above. The concentration of enzyme in each assay was approximately 5 nM in subunits. An aliquot of apoenzyme, stored at −196°C, was thawed at 4°C and appropriately diluted with assay buffer [5]. The reactions were started by mixing apoTK simultaneously with the remaining reagents ('immediate' assay) or after preincubation of apoTK with $MgCl_2$ and ThDP in assay buffer at 24°C for periods of 60 and 180 min ('preincubation' assay). In the latter case the reactions were started by addition of the remaining reagents.

Experimental data were analysed by nonlinear regression using either DNRPEASY [8] or GraphPAD Inplot computer programs.

## 3. Results and discussion

### 3.1. Sequence and sequence comparison

The entire sequence of both strands of the insert in pTrc99A/TK was determined. The coding region comprises 1869 nucleotides which are translated into a protein of 623 amino acid residues with a calculated subunit molecular weight of 67.8 kDa. Comparison of TK sequences from various species have revealed two regions of high homology [23]. The first region, termed the ThDP-binding motif [11], is located between amino acid residues 154 and 177 and is found in all known ThDP-dependent enzymes. The second region, located between residues 415 and 450, displays the highest degree of sequence similarity and has been shown to be characteristic of TK. This motif has been designated the TK motif [23].

Singleton and coworkers isolated various human TK cDNA clones from healthy controls and patients suffering from the neurodegenerative disorder Wernicke–Korsakoff (WK) syndrome. Their study clearly demonstrated that there is no genetic defect underlying this disorder [17], disproving the hypothesis that was originally proposed by Blass and Gibson [3]. Curiously, the nucleotide sequence deposited in GenBank by Singleton and coworkers differs from their consensus sequence, derived from controls and WK

patients, in several point mutations, four of which are nonsilent [17] The submitted sequence has been cloned in a plasmid and appeared intended for further studies, e.g. expression and site-directed mutagenesis. Although the characteristics of this construct have not yet been reported Singleton and coworkers have gone on to present results from site-directed mutagenesis studies [26], leaving open the question of whether unintended sequence variations are present in their expression system. It was our intention to minimize the number of mutations in our construct. This task was addressed by comparing our sequence with those of Singleton and coworkers and another complete cDNA sequence derived from the gene coding for fetal human TK [14]. The result is summarized in Table 1. In total, there are seven amino acid substitutions but, interestingly, in each case only one of the sequences differs whereas the others are identical at each of these positions, and the sequence we have determined always has a match in at least one of the other sequences. Furthermore, except for three synonymous substitutions, it is identical to the consensus sequence derived from the TK cDNA of controls and WK patients [17]. This sequence comparison further confirms that the sequence submitted to GenBank by Singleton and collaborators contains four nonsynonymous substitutions, an observation which is supported by an alignment including TK sequences from rodents [23].

In summary, this comparison indicates that there are no genuine variations between the reported human TK cDNA sequences. The construct used in this study appears to be identical to the consensus sequence without any undesired mutations, in contrast to the cDNA sequences reported previously [14, 17].

Table 1
Nucleotide and corresponding amino acid variants detected during sequence comparisons. Bases 1754–1755 and 1756–1758 belong to the same codons

| Base | This study | A[a] | B[b] | C[c] | Effect[d] |
|---|---|---|---|---|---|
| 88 | A | T | A | A | T30 → S |
| 91 | A | T | A | A | T31 → S |
| 93 | T | T | A | G | none |
| 137 | A | T | A | A | E46 → V |
| 465 | C | T | C | C | none |
| 534 | C | T | T | T | none |
| 582 | T | G | T | A | none |
| 1095 | T | C | T | T | none |
| 1209 | C | G | C | C | none |
| 1230 | T | C | C | C | none |
| 1276 | C | G | C | C | P426 → A |
| 1754 | C | C | C | A | T585 → K |
| 1755 | C | C | C | A | none |
| 1756 | C | C | C | A | |
| 1757 | A | A | A | C | H586 → T |
| 1758 | C | C | C | A | |
| 1759 | C | C | C | A | L587 → M |
| 1794 | G | A | G/A[e] | G | none |

[a] cDNA sequence submitted to GenBank by Singleton and coworkers [17].
[b] Consensus sequence derived from two WK patients and two healthy controls [17].
[c] Fetal human TK [14].
[d] Amino acids labelled in 1-letter code; the 'Effects' column shows the differences between the sequence of this study (left) and the one with a presumed mutation (right).
[e] Three sequences have a G and one an A in this position [17].

### 3.2. Expression and purification

Originally, human TK was expressed in pTrc99A (clone pTrc99A/TK). However, the modest yield rendered protein purification difficult. Cloning into pCL476 resulted in an N-terminal hexa-histidine tag fused to TK which simplified purification significantly. The synthesis of recombinant human TK in *E. coli* was induced by a rapid temperature shift from 30 to 42°C, which led to an inactivation of the thermolabile $\lambda$ repressor with the concomitant derepression of the strong $P_R$ and $P_L$ tandem promoters [10]. SDS-PAGE analysis of samples collected prior to induction and at various time points after the temperature shift showed that the expression level was still modest. Approximately 75% of the TK activity was in the soluble fraction (specific activity: $0.18 \pm 0.03$ U/mg, Table 2). However, the assay for TK activity does not differentiate between the recombinant and the native *E. coli* enzyme; only approximately 35% of the total activity was due to the human enzyme, estimated by comparing TK levels in *E. coli* expressing only native *E. coli* TK and cells expressing both *E. coli* TK and recombinant human TK. Assuming a specific activity of approximately 17 U/mg for homogeneous human TK [4] the recombinant protein represents only 0.21% of the total protein. Various different bacterial host cells, plasmids and growth conditions have been tested unsuccessfully for improvement of the expression level. Rare codon usage and unfavourable tRNA distribution could be reasons for the lack of high expression by this system [6]. Although the usage of rare codons may not account solely for low levels of expression it is evident that an mRNA with an unbalanced codon frequency will have different elongation rates for different regions of the sequence and different codons. The likelihood that a particular tRNA binds to the ribosomal A site depends strictly on the distribution of all other tRNAs and any disturbance of this distribution will have an influence on the speed of the elongation step [6]. The occurrence of a rare codon may stall the translation process long enough to terminate transcription prematurely. Different codon usage between the genes of human and *E. coli* transketolase is evident in various synonymous triplets, in particular in those coding for the amino acids phenylalanine, serine, tyrosine, proline, arginine, isoleucine, lysine, alanine, aspartate and glutamate and to a smaller degree in those coding for leucine, threonine, valine and glycine [22].

Despite the modest expression, recombinant human TK could be purified. The result of a typical purification is summarized in Table 2. Application of the soluble fraction directly to the nickel–IDA–Sepharose 6BFF column was tested, but some of the binding to the column was non-specific. Hydroxylapatite batch chromatography has been used previously for the purification of native transketolase from human erythrocytes, mainly as a means to remove the majority of the contaminating hemoglobin [4, 13]. These studies have shown that the native enzyme has a strong

Table 2
Purification of recombinant human TK

| Step | Volume (ml) | Protein (mg) | Activity (U) | Spec. Act. (U/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 315 | 6400 | 643 | 0.10 | 100 | 1 |
| Cleared lysate | 220 | 2684 | 490 | 0.18 | 76 | 1.8 |
| HA-unbound[a] | 127 | 1016 | 210 | 0.20 | 33 | 2 |
| HA-eluate[b] | 30 | 183 | 60 | 0.33 | 9 | 3.3 |
| Dialysate | 31 | 110 | 44 | 0.40 | 7 | 4 |
| $F^{c}_{max}$ | 2 | 1.2 | 16 | 13.5 | 2.5 | 135 |

[a] Fraction that did not bind to hydroxylapatite.
[b] Fraction that eluted from hydroxylapatite in 20% ammonium sulphate.
[c] Fraction 12 which eluted from the nickel–IDA–Sepharose 6BFF column.

affinity for hydroxylapatite and the activity could be eluted with a high recovery. In our study less than 60% of the activity bound to hydroxylapatite (Table 2) and only 12% of the activity was finally eluted in 20% ammonium sulphate. The fraction that did not bind to hydroxylapatite was applied to the nickel–IDA–Sepharose 6BFF and bound protein was eluted with an imidazole gradient as described in Section 2. Approximately 0.7% of the activity bound to the column, which leads to the proposition that the fraction that did not bind to hydroxylapatite is almost exclusively *E. coli* transketolase. The recombinant enzyme eluted from hydroxylapatite could be purified further by affinity chromatography on a nickel–IDA–Sepharose 6BFF column. The majority of the activity (65%) eluted in one single 2 ml fraction ($F_{max}$) with a specific activity of 13.5 U/mg (Fig. 2). SDS-PAGE (Fig. 3) shows that $F_{max}$ still contains a very small amount of contamination and this may explain in part why the specific activity is lower than the 17 U/mg obtained for pure human erythrocyte transketolase [4]. In total, 36 U of activity were recovered (2.1 mg transketolase protein).

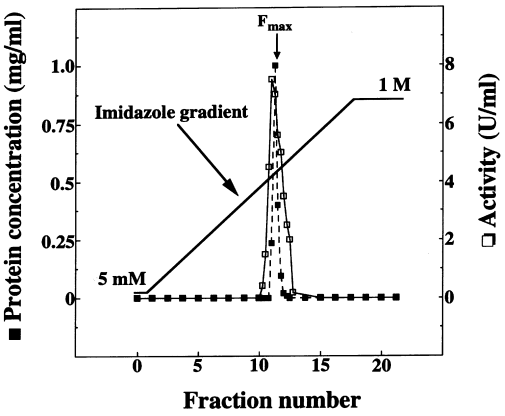


Fig. 2. Purification of recombinant human TK on a nickel–IDA–Sepharose 6BFF column. Recombinant human TK eluted from the nickel–IDA–Sepharose 6BFF column in a single sharp peak at an imidazole concentration of 650 mM.

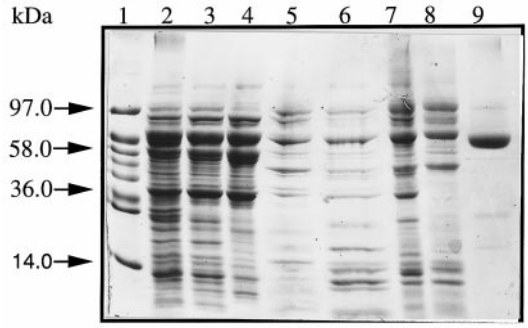


Fig. 3. SDS-PAGE analysis of the purification of recombinant human TK. Samples collected during the various stages of purification were analysed by SDS-PAGE. Lane 1 contains molecular weight markers as indicated in kDa. Lane 2, crude extract; lane 3, cleared lysate; lane 4, fraction that did not bind to hydroxylapatite; lanes 5 and 6, fractions eluted from hydroxylapatite; lane 7, pooled fractions from lanes 5 and 6 after dialysis; lane 8, fraction that did not bind to the nickel–IDA–Sepharose 6BFF column; lane 9, $F_{max}$ (fraction 12 eluted from the nickel–IDA–Sepharose 6BFF column).

### 3.3. Characterisation of recombinant human TK

A disadvantage of the described expression system is that the N-terminal hexa-histidine tag, which simplifies purification significantly, cannot be removed by protease treatment, resulting in a recombinant protein with several extra amino acids at the N-terminal end. The use of pCL476/TK as a source of TK, used in structure and function studies, is only justifiable if the kinetic properties of the recombinant enzyme agree with those of the native enzyme. The affinities for the major substrates R 5-P and X 5-P and both cofactors have been determined and compared with the values obtained for the native enzyme which has been purified and characterised previously in our laboratory [4, 32]. Table 3 summarizes the results; the $K_m$ values of the recombinant enzyme for the two major substrates are within the range of $K_m$ values reported for the native enzyme. TK is known to display hysteretic behaviour upon reconstitution of the apoenzyme with cofactors; addition of apoTK to an otherwise complete assay reaction mixture results in a variable lag phase in the reaction progress curve until steady-state activity is reached [5, 9, 25, 30]. Preincubation almost

Table 3
Kinetic parameters of recombinant and native human TK. Results are mean values ± S.D. ($n = 3$ for the recombinant enzyme)

| Parameter | Recombinant transketolase | Native transketolase |
|---|---|---|
| $V$[a] | 13.5 U/mg | 12–17 U/mg[b,c] |
| $K_m$ (X 5-P) | 0.27 ± 0.02 mM | 0.11 ± 0.08 mM[b], 0.49 ± 0.14 mM[c] |
| $K_m$ (R 5-P) | 0.51 ± 0.05 mM | 1.10 ± 0.04 mM[b], 0.53 ± 0.04 mM[c] |
| $K_m$ (ThDP) | 4.1 ± 0.8 μM (−)[e], 85 ± 16 nM (1 h)[f] | 2.3 ± 1.6 μM[d] (−)[e], 65 ± 14 nM[d] (1 h)[f] |
| $K_m$ ($Mg^{2+}$) | 2.52 ± 0.42 μM (−)[e] | 7.0 ± 0.6 μM[b] (−)[e] |

[a] Highest specific activity.
[b] Booth [4].
[c] Waltham [32].
[d] Tate and Nixon [30].
[e] (−): immediate assay.
[f] (1 h): 1 h preincubation assay.

abolishes this lag phase; however, at very low cofactor concentrations a preincubation time of several hours may be necessary [30]. Hence the apparent $K_m$ values of ThDP and $Mg^{2+}$ decrease with increasing incubation time; for ThDP the values range from 4.1 μM for the immediate assay to 15 nM for the 3 h preincubation assay; for $Mg^{2+}$ the values are 2.5 and 0.74 μM for the immediate (Table 3) and for the 1 h preincubation assay, respectively. The conditions required to resolve human TK [30] indicate that ThDP binds tightly to the enzyme. Therefore, it is likely that the $K_m$ values determined from the preincubation assays reflect the actual tight binding. Unfortunately, it is impracticable to incubate for even longer periods due to some instability of the apoenzyme [4, 5, 22, 30].

## 4. Conclusions

Although the described heterologous expression system yields only modest quantities of active recombinant human TK this protein could be separated from the host TK and highly purified. The amount of recovered activity is sufficient for kinetic and site-directed mutagenesis studies. However, in order to obtain enough protein for crystallization large culture volumes would need to be handled (≥50 l). It is, unfortunately, likely that the observed expression level is already at its limit for a bacterial host. So far, this is the first report of an heterologous expression system for any TK; it complements the previously reported homologous expression systems for *E. coli* and *S. cerevisiae* TK [28, 29]. These three systems provide the basis for detailed investigations of the similarities and differences between the three enzymes, which may lead to a comprehensive understanding of catalysis by TK and of its role in energy metabolism.

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