HAL
open science

Biochemical characterisation of the initial steps of the kennedy pathway in Trypanosoma brucei - the ethanolamine and choline kinases

Federica Gibellini, William N Hunter, Terry K Smith

To cite this version:

Federica Gibellini, William N Hunter, Terry K Smith. Biochemical characterisation of the initial steps of the kennedy pathway in Trypanosoma brucei - the ethanolamine and choline kinases. Biochemical Journal, 2008, 415 (1), pp.135-144. 10.1042/BJ20080435 . hal-00478985

HAL Id: hal-00478985

https://hal.science/hal-00478985v1

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Biochemical Journal Immediate Publication. Published on 20 May 2008 as manuscript BJ20080435

BIOCHEMICAL CHARACTERISATION OF THE INITIAL STEPS OF THE KENNEDY PATHWAY IN TRYPANOSOMA BRUCEI - THE ETHANOLAMINE AND CHOLINE KINASES

Federica Gibellini[1], William N. Hunter[1] & Terry K. Smith[2]*

[1]Division of Biological Chemistry & Drug Discovery, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

[2]Centre for Biomolecular Sciences, The North Haugh, The University, St. Andrews, KY16 9ST, Scotland, U.K.

*To whom correspondence should be addressed: Tel (0)1334-463412; email:tks1@st-andrews.ac.uk

Running title: Ethanolamine and choline kinases in *Trypanosoma brucei*

Key words: *Trypanosoma / ethanolamine kinase / choline kinase / specificity / Kennedy pathway*

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

ABSTRACT

Ethanolamine (EtN) and choline (Cho) are major components of the trypanosome membrane phospholipids, in the form of phosphatidylethanolamine (GPEtn) and phosphatidylcholine (GPCho). Ethanolamine is also found as an integral component of the glycosylphosphatidylinositol (GPI) anchor that is required for membrane attachment of cell surface proteins, most notably the variant surface glycoproteins. The *de novo* synthesis of GPEth and GPCho starts with the generation of phosphoethanolamine and phosphocholine by ethanolamine and choline kinases via the Kennedy pathway. Database mining revealed two putative choline/ethanolamine kinases (C/EKs) in the *T. brucei* genome, which were cloned, over-expressed, purified and characterised. *Tb*EK1 was shown to be catalytically active as an ethanolamine-specific kinase, i.e. no choline kinase activity. The Km values for ethanolamine and ATP were found to be 18.4 ± 0.9 μM and 219 ± 29 μM, respectively. *Tb*C/EK2, on the other hand, was found to be able to phosphorylate both ethanolamine and choline, even though choline was the preferred substrate, with a Km 80 times lower than that of ethanolamine. The Km values for choline, ethanolamine and ATP were; 31.4 ± 2.6 μM, 2.56 ± 0.31 mM and 20.6 ± 1.96 μM respectively. Further substrate specificity analysis revealed both *Tb*EK1 and *Tb*C/EK2 were able to tolerate various modifications at the amino group, with the exception of a quaternary amine for *Tb*EK1 (choline) and a primary amine for *Tb*C/EK2 (ethanolamine). Both enzymes recognized analogues with substituents on C2 but substitutions on C1 and elongations of the carbon chain were not well tolerated.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

INTRODUCTION

The zwitterionic glycerophospholipids phosphatidylcholine (GPCho) and phosphatidylethanolamine (GPEtn) are the two most abundant phospholipid species in eukaryotic cells and many bacteria [1]. In the parasitic protozoa *Trypanosoma brucei*, the aetiology of African sleeping sickness, GPCho accounts for approximately half of all phospholipids in both life cycle stages, whereas GPEtn forms between sixteen and twenty-one percent [2]. These phospholipids contribute an important structural role to the membrane and in addition determine membrane fluidity and cell surface charge. Their biosynthesis and utilisation is, not surprisingly, implicated in a variety of cellular processes [1]. In mammals GPCho is an important source of signalling molecules and the biosynthetic intermediate phosphocholine (PCho) has been reported to be a mitogen required for DNA synthesis induced by growth factors [3]. Due to its physical-chemical properties GPEtn has a tendency to form non-bilayer structures and its incorporation into membranes affects the processes of membrane trafficking and the folding, stabilization and incorporation of certain proteins into membranes [4]. Moreover, GPEtn is utilised in the biosynthesis of glycosylphosphatidylinositol (GPI), a complex glycolipid that anchors proteins to the exterior leaflet of eukaryotic plasma membranes [5-7]. This is particularly important for the bloodstream form of *T. brucei*, which expresses a dense cell-surface coat (10^7 molecules per parasite) of a GPI-anchored Variant Surface Glycoprotein (VSG). This coat prevents attack by components of the innate immune system and by undergoing antigenic variation, allows the parasite to evade a specific immune response by the host.

The biosynthesis of the GPI-anchor has been genetically [8-10] and chemically validated [11] as a drug target in bloodstream form *T. brucei*. Thus disruption of the biosynthesis of GPEtn could prevent the formation of mature GPI anchored VSG and lead to clearance of the parasite by the host's immune system.

Due to the major structural and functional roles of GPCho and GPEtn in *T. brucei*, disruption of their biosynthetic pathways is likely to interfere with the parasite biology in multiple ways and thus the enzymes involved become of interest as

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

possible novel targets for chemotherapy. Indeed it has been proposed that the mode of action of lysophospholipid analogues (miltefosine, edelfosine, ilmofosine) currently used against other trypanosomatid parasites, i.e. *Leishmania*, are involved in the inhibition of GPCho biosynthesis [12].

GPEtn and GPCho are *de novo* synthesised via two branches of the same metabolic pathway; the Kennedy pathway [13] (Fig. 1). Ethanolamine (EtN) and choline (Cho) are readily phosphorylated to generate phosphoethanolamine (P-EtN) and phosphocholine (P-Cho), which are subsequently activated to high-energy donors; CDP-EtN and CDP-Cho respectively, before they are coupled to diacylglyceride (DAG) to form GPEtn and GPCho. For this reason the two branches of the Kennedy pathway are often referred to as the CDP-Etn or CDP-Cho pathways. A second major pathway for the formation of GPEtn is via phosphatidylserine (GPSer) decarboxylation: GPSer, which is formed through the action of a GPSer synthase (PSS), is decarboxylated by a GPSer decarboxylase (PSD) to form GPEtn. In most eukaryotic cells GPCho can also be generated from GPEtn, by three methylation steps. However, according to the tri-Tryp genomes (http://www.genedb.org) these methyl-transferase genes are neither present in *T. brucei*, nor *T.cruzi*, but interestingly are present in *Leishmania*, this has been confirmed experimentally (Smith, unpublished data).

Although the presence of the Kennedy pathway was demonstrated in *T.brucei* [14] the constituent enzymes have never been characterized. Here, we report investigations into the first steps of both branches of the Kennedy pathway, involving molecular cloning, recombinant expression, purification and detailed kinetic characterisation of both ethanolamine and choline kinases (EK and CK) from *T. brucei*.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

EXPERIMENTAL PROCEDURES

Organisms and reagents

Trypanosoma brucei strain 427 was used as a source of genomic DNA. *Escherichia coli* strains DH5α, XL-1 blue and TOP10 (Invitrogen) competent cells were used for routine manipulation, and strain BL21(DE3)Gold (Novagen) for protein over-expression. All chemicals were purchased from either Sigma-Aldrich or Fluka. Restriction endonucleases and DNA modifying enzymes were from New England Biolabs or Promega. [2-^{3}H] Ethanolamine (50.0 Ci/mmol) and [methyl-^{3}H] Choline (82.0 Ci/mmol) were purchased from Amersham.

RNA isolation and cDNA synthesis

Total RNA was isolated from bloodstream form *T. brucei* using the RNeasy mini kit (Qiagen). A TbC/EK2 specific cDNA was generated and amplified using a mini-exon specific forward primer 5'-CGCGGATCCGAACGCTATTATTAGAACAGTTTCTGTAC-3' in combination with an open reading frame specific reverse primer 5'-GACTATACCCGTGGAACATCCG -3' specific for the sequence of TbC/EK2 using the SuperScript III One step RT-PCR kit with Platinum Taq (Invitrogen). The amplified cDNAs were purified (QIAquick PCR purification kit, Qiagen), ligated into pCR®-Blunt II TOPO® (Invitrogen) and sequenced.

Cloning of T. brucei EK1 gene and construction of the pET15b-TbEK1 expression vectors

Using the *Saccharomyces cerevisiae* ethanolamine kinase gene sequence as a query, two putative C/EK genes were identified in the *T. brucei* genome database (Sanger Centre, http://www.genedb.org) using tBlastN. The two putative open reading frames (Tb11.18.0017 *Tb*C/EK1, and Tb927.5.1140 *Tb*C/EK2) together with ~ 350bp of their 5'- and 3'- untranslated regions, were amplified from *T. brucei* strain 427 genomic DNA using Pfu DNA polymerase and the forward and reverse primers 5'-ATAAGTAAGCGGCCGCCCGCCTAAGTTAGAAGTTGCGCT-3' and 5'-ATAAGTAAGCGGCCGCTCCAATAGCTCCAGGGAAGGAAAGGGACG-3' for C/EK1; 5'-ATAAGTAA*GCGGCCGC*AAGTGCGTTGTGCAGGTCGGCGACGGT-3' and 5'-ATAAGTAA*GCGGCCGC*CGTTGGAAGGAGGAAAACGGCCGAGG-

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

3' for C/EK2. The resulting 2.1 Kb (C/EK1 and UTRs) and 3.7 Kb (C/EK2and UTRs) fragments were cloned into pCR®-Blunt-II TOPO® vector (Invitrogen). Clones were sequenced and compared with the annotated Gene Data Bank sequences. A BamHI restriction site, internal to the TbC/EK1 open reading frame was silenced by site directed mutagenesis using the forward primer 5'-GTGTTGAGGGGGATAAGCGAATCCATCGCATGGTTCAGC-3', the reverse primer 5'- GCTGAACCATGCGATGGATTCGCTTATCCCCCTCAACAC -3' and the QuickChange Site Directed Mutagenesis Kit (Stratagene).

The *Tb*C/EK1 open reading frame was PCR amplified from the TOPO® construct with *Pfu* polymerase using the forward and reverse primers 5'-GGAATTCCATATGATGGAGGTGGCTGTGGGGCAC-3' and 5'-CGCGGATCCGCGTTATGAAGATGCACTAAACTC-3' respectively. The amplicon was purified (QIAquick PCR purification kit, Qiagen) and ligated into pCR®-Blunt II TOPO® (Invitrogen) and sequenced. Using the NdeI and BamHI restriction site (underlined in primer sequences) the putative *TbC/*EK1 was ligated into the expression vector pET-15b (Novagen) modified with a Tobacco Etch Virus (TEV) protease cleavage site (in place of a thrombin cleavage site) *via* the same restriction sites generating the pET15bTEV-*Tb*C/EK1 construct. The same procedure was employed to ligate the putative *Tb*C/EK2 into the same vector using the forward and reverse primers 5'-GGAATTCCATATGATGGCATTACGACCGTTCCCG-3' and 5'-CGCGGATCCGCGTCAGGAAAGAAGCCCCTTCTC-3'.

The mutants of *TbEK1* D287A and D267A were generated by using the QuickChange Site Directed Mutagenesis Kit with the forward and reverse primers 5'-GGCGCACTGAAGATTATTG***c***CTTCGAGTATGCAAAACG-3' and 5'-CGTTTTGCATACTCGAAG***g***CAATAATCTTCAGTGCGCC-3' respectively for the D287A mutant and the forward and reverse primers 5'-GAGTACGTGCCACAATG***c***CCTGCTCAGCGGC-3' and 5'-GCCGCTGAGCAGG***g***CATTGTGGCACGTACTC-3' respectively for the D267A mutant (the lower case letters in the primers highlight the mutated bases). All DNA sequencing was performed by The Sequencing Service (College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Expression and Purification of TbC/EK1 and TbC/EK1

Overproduction and sample preparation

The pET15bTEV-*Tb*C/EK1 and the pET15bTEV-*Tb*C/EK2 constructs were transformed in BL21(DE3)Gold cells and clones selected on LB-agar plates containing carbenicillin (100 μg/ml). Single colonies were grown at 37°C in LB medium containing carbenicillin (50 μg/ml) until OD_{600nm} was between 0.5 and 0.7, at which point recombinant protein expression was induced with 1mM isopropyl-β-D-thiogalactopyranoside and grown for 16 hours at room temperature. Cells were harvested by centrifugation (3500 *g*, 20 min, 4°C) and suspended in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). Cells were lysed in the presence of Dnase I, either by sonication or using a French press and the lysate cleared by centrifugation (35,000 *g*, 30 min, 4°C).

Purification and gel filtration

The cleared lysate was applied to a 1 ml HisTrap™ FF crude column or a 5 ml HisTrap ™ column (GE-Healthcare) preloaded with Ni^{2+}. Unbound proteins were removed by washing the column with 15 column volumes of buffer A, while *Tb*C/EK1 and *Tb*C/EK2 were eluted with an imidazole gradient in the same buffer.

Fractions containing *Tb*C/EK1 or *Tb*C/EK2 were pooled, dialysed against 50 mM Tris-HCl pH 8, 300 mM NaCl and further purified through a Superdex200 16/60 gel filtration column (GE-Healthcare), run in the same buffer at a rate of 1 ml/min. Low and High Molecular Weight Calibration Kits (GE-Healthcare) were used to determine the molecular weight and the oligomeric state of the proteins. Purity of protein samples was assessed by SDS-PAGE and matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (Proteomics Facility, College of Life Sciences, University of Dundee).

Assay of TbC/EK activity

Ethanolamine/choline kinase activity was measured by a spectrophotometric coupled assay as described previously [15]. The assay contained 50 mM MOPS pH 7.8, 150

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

mM KCl, 6 mM $MgCl_2$, 0.5 mg/ml BSA, 1 mM phosphoenolpyruvate, 0.5 mM NADH, 7 units/ml pyruvate kinase, 32 units/ml lactate dehydrogenase, 2 μg/ml *Tb*C/EK1 or 1 μg/ml *Tb*C/EK2. Concentrations of ATP and ethanolamine (or choline) were varied and the rate of the reaction monitored by the decrease in absorbance at 340 nm (consequence of NADH oxidation) in a Shimadzu UV-1601 Spectrophotometer. Enzyme Kinetics, SigmaPlot (SPSS Inc.), was used to analyse and fit the kinetic data.

The pH optimum was assessed by measuring *Tb*C/EK1 and *Tb*C/EK2 activities at various pHs: sodium acetate (pH 5.0), Bis-Tris propane or MOPS (pH 6.5), Tris-HCl (pH 7.0, 8.0, 8.5), MOPS (pH 6.0, 7.0, 7.4, 7.8) and glycine (pH 9.0). Various ethanolamine and choline analogues were tested as substrates at least in triplicate on 96 wells plates using a SpectraMAX 340PC plate reader (Molecular Devices) and those deemed as non-substrates were tested as inhibitors by preincubation at 1mM for 5 min prior to the addition of ethanolamine or choline (0.1mM).

Direct ethanolamine and choline kinase activity assays were performed by assessing the production of phosphoethanolamine or phosphocholine, using a modified method of Kim *et. al.,* [16]. Briefly, 2 μg of purified protein is incubated with a reaction mixture (total volume 50 μl) of 100 mM MOPS pH 7.8, 6 mM $MgCl_2$, 5 mM ATP, with either 2 mM ethanolamine and 0.2 μCi [^{3}H]ethanolamine or 2 mM choline and 0.2 μCi [^{3}H]choline at 37°C for 20 minutes and quenched by boiling at 100°C for 5 min. Substrates and products were separated by HPTLC using silica 60 plates with methanol: 0.6% sodium chloride: ammonium hydroxide (10:10:1, v/v) as solvent. Radiolabelled species were detected by fluorography at -70°C after spraying with En^{3}hance and using Kodak XAR-5 film with an intensifying screen. Unlabelled ethanolamine and phosphoethanolamine standards were run in parallel and visualized by spraying with ninhydrin (0.2% in water-saturated butan-1-ol). Unlabelled phosphocholine was run in parallel and visualised by iodine staining.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

RESULTS AND DISCUSSION

Genomic identification and cloning of *Tb* C/EK1 and 2

Two putative Cho/EtN kinases were identified in the *T. brucei* genome database (Sanger Centre); these putative ORFs were PCR-amplified from genomic DNA, cloned and sequenced. Two potential initiating methionines were detected in the *Tb*C/EK2 ORF, this ambiguity was resolved by amplification and sequencing of the first ~350 bp of the RT-PCR product using total RNA, a forward primer against the mini-exon and a reverse primer specific for the *Tb*C/EK2 ORF. From the cDNA sequence (supplementary Fig 1) the second methionine is identified as the initiating residue. Interestingly there seems to be extra 'stuffer' sequence (65 bp) between this and the mini-exon (supplementary Fig 1), this may represent some form of regulatory element, a concept we are currently investigating.

The predicted molecular masses of the deduced amino acid sequences are 49.1 kDa (431 amino acids) for *Tb*C/EK1 and 70.1 kDa (628 amino acids) for *Tb*C/EK2. An alignment of the translated sequences of *Tb*C/EK1 and 2, with previously characterised or putative C/EKs from other organisms (Figure 2) shows significant similarities. Even though the amino-terminal region is not generally well conserved amongst C/EKs, the carboxy-terminal portion contains two highly conserved regions. The Brenner's motif (Figure 2) has been identified in many enzymes catalysing phosphotransfer reactions, such as protein kinases and atypical kinases (the superfamily that includes the choline/ethanolamine kinase family) [17]. The second conserved cluster (Figure 2) is found specifically in members of the choline/ethanolamine kinase family and is referred to as the choline kinase motif. Previous site-directed mutagenesis studies and structural studies in *Caenorhabditis elegans* CKα2 [18,19], and the analysis of the crystal structures of human CKα [20] highlighted the importance of several of the amino acids in substrate binding. Of particular importance are the two Asp residues marked by an asterisk in the alignment (Figure 2). These residues are involved in the binding of ATP and the stabilization of the transition state of the reaction and their mutation leads to complete enzyme inactivation [18,20].

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Not surprisingly, *Tb*C/EK1 and 2 are most closely related to C/EKs from other kinetoplastids. In the region of the alignment shown in Figure 2 *Tb*C/EK1 is 63% identical to a putative C/EK from *T. cruzi* and *Tb*C/EK2 is 40% identical to a putative C/EK from *L. major*. The similarity towards previously characterised C/EKs from human, yeast, *C.* elegans and *Plasmodium falciparum* is much lower (data not shown), with the exception of the Brenner's and the choline kinase motives. The *Tb*C/EK1 and *Tb*C/EK2 sequences have been submitted to GenBank Nucleotide Sequence Database with Accession numbers AM939568 and AM939569 respectively.

Recombinant expression and purification of *Tb*C/EK1 and *Tb*C/EK2

In order to confirm that the putative *Tb*E/CK1 and *Tb*E/CK2 are catalytically active, the two ORFs were cloned into the vector pET-15bTEV. This vector encodes an N-terminal hexa-histidine epitope-tag followed by a TEV protease site, which allowed purification of the recombinant proteins via nickel-ion affinity chromatography (Fig. 3A and B). MALDI mass spectrometry of the recombinant *Tb*C/EK1 and *Tb*C/EK2 were very close to their theoretical masses of 51655 and 72614 Da respectively. The proteins *Tb*C/EK1 and *Tb*C/EK2 were further purified by gel-filtration (Fig. 3C). Elution from the gel filtration column showed that both *Tb*C/EK1 (filled arrow) and *Tb*C/EK2 (empty arrow) exhibit a dimeric state in solution (Fig. 3D). This is similar to most of the choline/ethanolamine kinases from other sources [21-23] with the notable exception of the monomeric *P. falciparum* choline kinase [15]. Typical yields were 10 mg and 15 mg per litre of bacterial culture for *Tb*C/EK1 and *Tb*C/EK2, respectively. TbC/EK1 was found to lose activity over time, being completely lost after a couple of days. It is also prone to precipitation upon freeze thawing, and therefore assays were preformed on freshly prepared batches. TbC/EK2 on the other hand was stable and freeze thawing did not lead to loss of activity or precipitation.

Kinetic analysis of *Tb*C/EK1 and *Tb*C/EK2

Previously characterised choline/ethanolamine kinases from various sources [15,21-25] have exhibited different specificity towards the two substrates; choline and ethanolamine. It was therefore necessary to test the activity of the purified proteins in order to understand if they were able to catalyse the phosphorylation of both choline and ethanolamine or if they showed selectivity to each branch of the Kennedy pathway.

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

*Tb*C/EK1 was found to be able to catalyse the formation of phosphoethanolamine from ethanolamine, in an ATP and magnesium dependant manner (Fig. 4A and B). However the enzyme was unable to catalyse the formation of phosphocholine from choline, in the presence of ATP and magnesium (Tables 1 and 2). This restricts the specificity of this kinase to the CDP-ethanolamine branch of the Kennedy pathway and for this reason *Tb*C/EK1 was renamed *Tb*EK1.To our knowledge this is the first ethanolamine-specific kinase to be recombinantly expressed, purified to homogeneity and extensively characterised.

Kinetic analysis showed that *Tb*EK1 displayed a pH optimum at pH 8.0 (Fig. 5A), with a Km of 18.4 ± 0.9 μM for ethanolamine (Fig. 5B) and a Km of 219 ± 29 μM for ATP (Fig. 5C), which is in reasonable agreement with a previously reported Km value of 2.75 μM for ethanolamine kinase activity from crude trypanosome homogenates [14]. The Vmax of *Tb*EK1 is 7.62 ± 0.07 μmol/min/mg, corresponding to a kcat of 6.56 s^{-1}(Table 1), this turnover rate / specific activity compares favourably with the previously reported activities of 346 nmol/min/mg for the yeast EK [16] and 10.73 μmol/min/mg for a soybean EK [25]. Two catalytically inactive mutants of *Tb*EK1 were generated by substitution of Asp267 and Asp286 (Fig. 4B and C) with Ala, thus confirming the crucial importance of these two residues in the catalytic mechanism of this kinase.

*Tb*C/EK2, on the other hand, was found to be able to phosphorylate both ethanolamine (Figure 6B) and choline (Figure 6A and 6C), even though choline was the preferred substrate (Table 1 and 2), with a Km 80 times lower than that of ethanolamine. *TbC/*EK2 was also dependent upon ATP and magnesium for activity (Figure 6B and 6C) with a pH optimum of 7.8 (Fig. 7A). Kinetic analysis showed that *TbC/*EK2 displayed Km values for choline, ethanolamine and ATP of 31.4 ± 2.6 μM, 2.56 ± 0.31 mM and 20.6 ± 1.96 μM respectively with a Vmax of 10.67 ± 0.18 μmol/min/mg and a kcat of 12.91 s^{-1} (Figures 7B-D, Table1). This value is in the range of activities reported for other eukaryotic C/EKs; and more than ten times higher than the choline kinase from *P. falciparum* [15].

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Since the *Tb*C/EK2 Km value for ethanolamine is several orders of magnitude higher than *Tb*EK1 and the fact that levels of ethanolamine in human plasma are in the micromolar range [26], one can reasonably assume that *Tb*EK1 accounts for the vast majority of the ethanolamine-phosphate formation in the trypanosome. There were no differences in the kinetics between the His-tagged and untagged (TEV cleaved) versions of either *Tb*EK1 or *Tb*C/EK2, thus the tagged versions were used as this decreases preparation time and increases stability, especially important for *Tb*EK1.

Acceptor specificity of *Tb*EK1 and *Tb*C/EK2

The ability of the two enzymes to utilise chemically modified substrates for the phosphotransfer reaction was investigated using a variety of ethanolamine and choline analogues, with modifications either at the amino- or the hydroxyl- moieties, or with additional substituents on the two-carbon backbone. Each analogue was compared with ethanolamine (for *Tb*EK1) or choline (for *Tb*C/EK2) at a constant substrate concentration (1 mM) and a fixed, saturating concentration of ATP to give a relative rate (ethanolamine, choline = 100) (Tables 2-4).

*Tb*EK1 was able to tolerate various modifications at the amino group, including secondary and tertiary amino modifications despite having been shown earlier not to process choline, (Table 2). As shown in Table 3 single methyl and ethyl substituents on the carbon backbone at C2 (*R*(-)2-amino-1-butanol, *S*(+)2-amino-1-butanol, *R*(-)2-amino-1-propanol, *S*(+)2-amino-1-propanol) are well tolerated, but the introduction of a second methyl group at C2 (2-amino-2-methyl-1-propanol) or a negatively charged carboxyl group (L-serine) resulted in greatly reduced or non detectable activity, respectively. Methyl substitutions on C1 (*R*(-)1-amino-2-propanol, *S*(+)1-amino-2-propanol) diminished the ability of the compound to act as a substrate, but to varying degrees in a stereospecific fashion. The increase of the carbon backbone length by one methylene unit (3-amino-1-propanol) led to a pronounced decrease in activity, whereas the increase by an ethylene unit (4-amino-1-butanol), completely eliminated substrate activity.

*Tb*C/EK2 was able to accept analogues modified at the amino group as substrates of the reaction, in fact secondary and tertiary amino modifications were better than choline. Notably, of the compounds tested, ethanolamine was the poorest acceptor substrate (Table 2), these findings are in contrast to those with CK from brewer's yeast [24], where N-methylethanolamine and N,N-dimethylethanolamine

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

were as poor substrates as ethanolamine. Compounds with methyl and ethyl alterations on C2 are good substrates only in the (*S*) configuration; *R*(-)2-amino-1-butanol and *R*(-)2-amino-1-propanol are much poorer substrates, as well as 2-amino-2-methyl-1-propanol, which bears two methyl groups at C2 (Table 3). A reduction of activity is seen with the elongation of the carbon chain, but it is not abolished even when increased by an ethylene unit (4-amino-1-butanol). The introduction of a negative charge (L-serine) as well as the introduction of substituents at C1 (*R*(-)1-amino-2-propanol, *S*(+)1-amino-2-propanol) are not tolerated (Table 3). That the enzyme is relatively more tolerant of substituents on C2 is in agreement with the previous studies on the yeast orthologue [24]. As expected, modifications at the hydroxyl group (Table 4) resulted in no detectable reactivity for both *Tb*EK1 and *Tb*C/EK2.

Analogues deemed as non-substrates were tested as potential inhibitors by pre-incubation at 1 mM for 5 min prior to the addition of ethanolamine or choline (0.1 mM) to *Tb*EK1 and *Tb*C/EK2 respectively. However, none of the potential inhibitory analogues showed any inhibitory effect (<5 %) on either *Tb*EK1 or *Tb*C/EK2 activity.

Previous studies in *T. brucei* [14] demonstrated that ethanolamine analogues, such as N-methylethanolamine, N-ethylethanolamine, N,N-dimethylethanolamine, *S*-(+)-2-amino-1-butanol, *R*-(-)-2-amino-1-butanol, can be recognized by the ethanolamine transport system, whereas choline is not. There was ambiguity as to whether these compounds were acting as inhibitors of the transporter or merely competing with ethanolamine to be transported into the cell. If the latter case were true, our results suggest these analogues could be phosphorylated by either *Tb*EK1 and/or *Tb*C/EK2, and possibly proceed further in the Kennedy pathway leading to the formation of unusual phosphatidylethanolamine analogues, a concept we are currently pursuing.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

CONCLUSIONS

African sleeping sickness is a neglected disease, which urgently requires new drug therapies. Characterising fundamental differences in the biochemical pathways and enzymes therein, of the parasite and its mammalian host is an important first step towards this goal. Thus, we present here for the first time, the identification and detailed molecular characterization of the two putative choline/ethanolamine kinases in *T. brucei*. *Tb*EK1, is committed to only the CDP-ethanolamine branch of the Kennedy pathway since it is unable to phosphorylate choline; *Tb*C/EK2 on the other hand, was able to phosphorylate choline much more efficiently than ethanolamine. The substrate specificities for each kinase were determined, providing crucial information on the structural requirements for ligand recognition.

Analysis of the roles of *Tb*EK1 and *Tb*C/EK2 in parasite survival will be crucial to investigate their potential as drug targets for the development of new chemotherapeutic agents against African sleeping sickness and perhaps other protozoan parasites. The similarities in ligand recognition displayed by *Tb*EK1 and *Tb*C/EK2 may guide the design of inhibitors able to target both enzymes at once. Further work will characterise and assess the importance of all steps of the two branches of the Kennedy pathway, as well as their functional role in the biology of the trypanosome.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

ACKNOWLEDGEMENTS

This work was supported by a Wellcome Trust Senior Research Fellowship 067441(TKS), Wellcome Trust grants 082596 and 083481 (WNH) and a Wellcome Trust PhD studentship (FG).

FOOTNOTES

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession numbers AM939568 and AM939569.

ABBREVIATIONS

The abbreviations used are: GPEtn, phosphatidylethanolamine; GPCho, Phosphatidylcholine; GPSer, Phosphatidylserine ; VSG, variant-surface glycoprotein; ORF, open reading frame; UTR, untranslated region; HPTLC, high performance thin layer chromatography; C/EK, Choline/Ethanolamine Kinase; ECT, Ethanolamine-phosphate Cytidyltranspherase; EPT, Ethanolamine Phosphotransferase; EtN, Ethanolamine; GPI, Glycosylphosphatidylinositol; MADLI, matrix-assisted laser desorption/ionization; PSD, Phosphatidylserine Decarboxylase; PSS, Phosphatidylserine Synthase; TEV, Tobacco Etch Virus.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

REFERENCES

[1] Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: Why are there so many lipids? Annu. Rev. Biochem. **66**:199-232

[2] Patnaik, P. K., Field, M.C., Menon, A.K., Cross, G.A.M., Yee, M.C. and Bütikofer, P. (1993) Molecular species analysis of phospholipids from *Trypanosoma brucei* bloodstream and procyclic forms. Mol. Biochem. Parasitol. **58**: 97-105

[3] Cuadrado, A., Carnero, A., Dolfi, F., Jiménez B. and Lacal, J.C. (1993) Phosphorylcholine: a novel second messenger essential for mitogenic activity of growth factors. Oncogene, **8**: 2959-2968

[4] Bürgemaister, M., Birner-Grünberger R., Heyn, M. and Daum, G. (2004) Contribution of different biosynthetic pathways to species selectivity of aminoglycerophospholipids assembled into mitochondrial membranes of the yeast *Saccharomyces cerevisiae*. Biochim. et Biophys. Acta **1686**:148-160

[5] Imhof, I., Canivenc-Gansel, E., Meyer, U. and Conzelmann, A. (2000) Phosphatidylethanolamine is the donor of the phosphorylethanolamine linked to the α-1,4-linked mannose of yeast GPI structure. Glycobiology **10**: 1271-1275

[6] Menon, A.K., Eppingerl, M., Mayor, S. and Schwarz, R. (1993) Phosphatidylethanolamine is the donor of the phosphoethanolamine group in trypanosome glycosyl-phosphatidylinositol. EMBO J. **12**: 1907-1914

[7] Menon, A.K., and Stevens, V.L. (1992) Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein J. Biol. Chem. **267**: 15277-15280

[8] Nagamune, K., Nozaki, T., Maeda, Y., Ohishi, K., Fukuma, T., Hara, T., Schwarz, R.T., Sutterlin, C., Brun, R., Riezman, H. and Kinoshita, T. (2000) Critical roles of glycosylphosphatidylinositol for *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA **97**: 10336-10341.

[9] Chang, T., Milne, K.G., Guther, M.L.S., Smith, T.K. and Ferguson, M.A.J. (2002) Cloning of the *Trypanosoma brucei* and *Leishmania major* genes encoding the GlcNAc-phosphatidylinositol de-N-acetylase of biosynthesis that is essential to the African sleeping sickness parasite. J. Biol. Chem. **277**: 50176-50182.

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

[10] Ferguson, M.A.J., (2000) Glycosylphosphatidylinositol biosynthesis validated as a drug target for African sleeping sickness. Proc. Natl. Acad. Sci. USA **97**: 10673-10675

[11] Smith, T.K., Crossman, A., Brimacombe, J.S. and Ferguson M.A.J. (2004) Chemical validation of GPI biosynthesis as a drug target against African sleeping sickness. EMBO J. **23**: 4701-4708

[12] Urbina, A. J. (2006) Mechanisms of action of lysophospholipid analogues against trypanosomatid parasites. Trans. R. Soc. Trop. Med. Hyg. **100**: S9-S16

[13] Kennedy E.P., and Weiss, S.B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. **222**:193-214

[14] Rifkin, M.R., Strobos, C.A.M. and Fairlamb, A.H. (1995) Specificity of ethanolamine transport and its further metabolism in *Trypanosoma brucei*. J. Biol. Chem. **270**: 16160-16166

[15] Choubey, V., Guha, M., Maity, P., Kumar, S., Raghunandan R., Maulik, P.R., Mitra, K., Halder, U.C. and Bandyopadhyay, U. (2006) Molecular characterization and localization of *Plasmodium falciparum* choline kinase. Biochim. Biophys. Acta **1760**: 1027-1038

[16] Kim, K., Kim, K.H., Storey, M.K., Voelker, D.R. and Carman, G.M. (1999) Isolation and characterization of the *Saccharomyces cerevisiae* EKI1 gene encoding ethanolamine kinase. J. Biol. Chem. **274**: 14857-14866

[17] Scheef, E.D. and Bourne, P.E. (2005) Structural evolution of the protein kinase-like superfamily PLoS Comput. Biol. **1**: e49

[18] Yuan, C. and Kent, C. (2004) Identification of critical residues of choline kinase A2 from *Caenorhabditis elegans*. J. Biol. Chem. **276**: 17801-17809

[19] Peisach, D., Gee, P., Kent, C. and Zhaohui X. (2003) The crystal structure of choline kinase reveals a eukaryotic protein kinase fold. *Structure*, **11**: 703–713

[20] Malito, E., Sekulic, N., Cun, W., Too, S., Konrad, M. and Lavie, A. (2006) Elucidation of human choline kinase crystal structures in complex with the products ADP or phosphocholine. J. Mol. Biol. **364**: 136–151

[21] Gee, P., and Kent, C., (2003) Multiple isoforms of choline kinase from *Caenorhabditis elegans*: cloning, expression, purification, and characterization. Biochim. Biophys. Acta **1648**: 33-42

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

[22] Ayoama., C., Liao, H. and Ishidate, K. (2004) Structure and function of choline kinase isoforms in mammalian cells. Prog. Lipid Res. **43**: 266-281

[23] Kim, K., Voelker, D.R., Flocco, M.T. and Carman, G.M. (1998) Expression, purification, and characterization of choline kinase, product of the CKI gene from *Saccharomyces cerevisiae*. J. Biol. Chem. **273**: 6844-6852

[24] Clary, G.L., Tsai, C.F. and Guynn, R.W. (1986), Substrate specificity of choline kinase. Arch. Biochem. Biophys. **254**:214-221

[25] Wharfe, J. and Harwood, J.L. (1976) Lipid metabolism in germinating seeds. Purification of ethanolamine kinase from soya bean. Biochim Biophys Acta. **575**: 102-11

[26] Baba, S., Watanabe, Y., Geiyo, F. and Arakawa, M. (1984) High-performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure. Clin. Chim. Acta **136**: 49-56

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

FIGURE LEGENDS

Figure 1. Pathways for the biosynthesis of the aminoglycerophospholipids phosphatidylcholine (GP-Choline) and phosphatidylethanolamine (GP-Ethanolamine). The Kennedy pathways shown include the relevant steps discussed in the text along with their genedb numbers (www.genedb.org); CK, choline kinase (Tb 11.18.0017 / Tb 927.5.1140); CCT, CTP: phosphocholine cytidyltransferase (Tb 10.389.0739); CPT, diacylglycerol: CDP-choline choline-phosphotransferase (Tb 10.6k15.1570); EK, ethanolamine kinase (Tb 11.18.0017 / Tb 927.5.1140); ECT, CTP: phosphoethanolamine cytidyltransferase (Tb 11.01.5730); EPT, diacylglycerol: CDP-ethanolamine ethanolamine-phosphotransferase (Tb 10.615.1570); GPSer, phosphatidylserine; GPEtn, phosphatidylethanolamine; GPCho, phosphatidylcholine; PSD, phosphatidylserine decarboxylase(Tb 09.211.1610); PEMT, phosphatidylethanolamine methyltransferases. The dashed box indicates methyltransferase enzymes which are neither present in *T. brucei* nor *T.cruzi*, but are present in *Leishmania.*

Figure 2. A Tcoffee alignment of the predicted amino acid sequences of *Trypanosoma brucei* C/EK 1 and 2 with choline and ethanolamine kinases of other eukariotic organisms: *Trypanosoma cruzi* (Q4E3A9), *Leishmania major* (Q4FYH6, Q4FWX2) and *Homo sapiens* (Q9HBU6). Residues are shaded according to percentage similarity using the software Jalview 2.08.1. The boxes highlight the choline kinase motif and the Brenner's phosphotransferase motif. The two Asp residues marked by an asterisk were mutated in *Tb*EK1 to generate the catalytically inactive mutants D267A and D286A.

Figure 3. Expression and purification of recombinant *Tb*C/EK1 and *Tb*C/EK2 in *E. coli*.

(A) *Tb*C/EK1 protein samples from each purification step were separated on a 4-12 % SDS-PAGE gel and stained with Coomassie brilliant blue. Lane1, Total *E. coli* cellular protein after induction; Lane 2, Cleared cell lysate; Lane 3, nickel column wash at 20 mM imidazole; Lane 4, nickel column wash at 50 mM imidazole; Lanes 5-8 subsequent elutions from the nickel column at 250 mM imidazole.

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

(B) *Tb*C/EK2 protein samples from each purification step were separated on a 10 % SDS-PAGE gel and stained with Coomassie brilliant blue. Lane1, Cleared *E. coli* cell lysate; Lanes 2-4 washes; Lanes 5-8 peak of *Tb*C/EK2 elution at 220 mM imidazole.
(C) Protein samples after gel filtration separated on a 4-12% (*Tb*C/EK1, Lane 1) and a 10% (*Tb*C/EK2, Lane 2) SDS-PAGE gel.
(D) Determination of native molecular masses of *Tb*C/EK1 and *Tb*C/EK2. The elution from the gel filtration column indicates both *Tb*EK1 (black arrow) and *Tb*C/EK2 (white arrow) are present as dimers. The following proteins were used as standards: (a) Ferritin (440 KDa), (b) Conalbumin (75 KDa) ,
(c) Carbonic anhydrase (29 KDa), (d) Ribonuclease A (13.7 KDa)

Fig. 4. Analysis of phosphoethanolamine formation by *Tb*C/EK1
(A) Reaction catalysed by *Tb*EK1. EtN, ethanolamine; EtN-P, phosphoethanolamine. (B) The substrate ethanolamine and the product phosphoethanolamine of the reaction were separated by HPTLC as described in *Experimental*. Lanes 1-6, autoradiography detection. Lane 1, [^{3}H]ethanolamine standard; Lane 3, standard reaction with *Tb*C/EK1; Lanes 2 and 4, no enzyme or no Mg^{2+} added to the reaction mixture respectively (negative controls); standard reaction with *Tb*EK1 mutants D267A and D286A (lanes 5 and 6 respectively); Lane 7, ethanolamine (EtN) standard; Lane 8, phosphoethanolamine (EtN-P) standard detected by ninhydrin. (C) Enzymatic activities of wild-type *Tb*EK1 and mutants D267A and D286A assessed by spectrophotometric coupled assay as described in *Experimental*. The insert shows an SDS-PAGE of the purified proteins (wild-type and mutants).

Fig. 5 Kinetic analysis of *Tb*C/EK1
(A) Ethanolamine kinase activity was measured spectrophotometrically as a function of pH as described in *Experimental.* Data are presented as mean ± SD from three measurements. (B,C) Determination of *Tb*EK1 Michaelis-Menten constants for ethanolamine and ATP (inserts Lineweaver-Burk plots). (B) ATP concentration was held constant (5 mM) while ethanolamine concentration was varied. (C) Ethanolamine concentration was held constant (2 mM) while ATP concentration was varied.

Fig. 6. Analysis of phosphocholine formation by *Tb*C/EK2

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

(A) Reaction catalysed by *Tb*C/EK2. Cho, choline; Cho-P, phosphocholine. (B) The substrate ethanolamine (EtN) and the product phosphoethanolamine (EtN-P) of the reaction were separated by HPTLC as described in *Experimental.* Lanes 1-7, ninhydrin detection. Lane 1, ethanolamine standard; Lane 2, phosphoethanolamine standard; Lane 3, standard reaction with *Tb*C/EK2; Lanes 4-6 no Mg^{2+}, no ethanolamine or no ATP added to the reaction mixture, respectively (negative controls). (C) The substrate choline and the product phosphocholine of the reaction were separated by HPTLC as described in *Experimental.* Lanes 1-6, autoradiography detection. Lane 7, detection by iodine staining. Lane 1, [^{3}H]-choline standard; Lane 2, standard reaction with *Tb*EK2; Lanes 3-6 no enzyme, no Mg^{2+}, no [^{3}H]-choline or no ATP added to the reaction mixture, respectively (negative controls); Lane 7, phosphocholine standard visualised by iodine staining.

Fig. 7 Kinetic analysis of *Tb*C/EK2

(A) Choline kinase activity was measured spectrophotometrically as a function of pH as described in *Experimental.* Data are presented as mean ± SD from three measurements. (B,C,D) Determination of TbC/EK2 Michaelis-Menten constants for choline, ethanolamine and ATP (inserts Lineweaver-Burk plots). ATP concentration was held constant (5 mM) while choline (B) or ethanolamine (C) concentrations were varied. (D) Choline concentration was held constant (2 mM) while ATP concentration was varied.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Stage 2(a) POST-PRINT

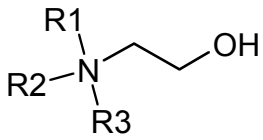
Table 1: Kinetic values for recombinantly expressed and purified TbEK1 and TbC/EK2

Enzyme	Vmax (μmol/ min/mg protein)	Km Cho (μM)	Km Etn (μM)	Km ATP (μM)	kcat (s^{-1})	kcat/Km Cho (s^{-1} mM^{-1})	kcat/Km Etn (s^{-1} mM^{-1})	kcat/Km ATP (s^{-1} mM^{-1})
Tb EK1	7.62 ± 0.07	0.0 ± 0.0	18.4 ± 0.9	219.2 ± 29	6.56	-	356.53	29.93
Tb CK2	10.67 ±0.18	31.4 ± 2.6	2600 ± 310	20.6 ± 1.96	12.91	411.2	5.04	626.86

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Table 2: Substrate specificity of *Tb*EK1 and *Tb*C/EK2 to utilise analogues modified at the amino group of the acceptor substrate.

R1	*R2*	*R3*	*Compound*	*TbEK1 Relative rate (a)*	*S.D.*	*TbC/EK2 Relative rate (b)*	*S.D.*
$-CH_3$	$-CH_3$	$-CH_3$	Choline	0.03	1.59	**100.00**	**7.97**
-H	-H	-H	Ethanolamine	**100.0**	**1.65**	33.34	3.34
-H	-H	$-CH_3$	N-Methylethanolamine	106.58	9.18	135.90	3.64
-H	$-CH_3$	$-CH_3$	N,N-Dimethylethanolamine	76.54	4.86	153.33	2.77
-H	-H	$-CH_2CH_3$	N-Ethylethanolamine	97.38	1.59	150.57	8.40
-H	$-CH_2CH_3$	$-CH_2CH_3$	N,N-Diethylethanolamine	47.75	3.98	155.92	4.32
-H	-H	$-CH_2CH_2OH$	Diethanolamine	88.26	0.78	138.86	6.30



(a) Analogues are compared with ethanolamine at the constant concentration of 1 mM and a saturating concentration of ATP to give a relative rate (ethanolamine = 100)

(b) Analogues are compared with choline at the constant concentration of 1 mM and a saturating concentration of ATP to give a relative rate (choline = 100)

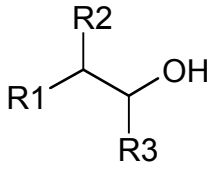
Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Table 3: Substrate specificity of *Tb*EK1 and *Tb*C/EK2 to utilise analogues modified by extra substitutents on the carbon backbone of the acceptor substrate.

R1	*R2*	*R3*	*Compound*	*TbEK1 Relative rate (a)*	*S.D.*	*TbC/EK2 Relative rate (b)*	*S.D.*
$-N^{+}(CH_3)_3$	-H	-H	Choline	0.03	1.59	**100.00**	**7.97**
$-NH_2$	-H	-H	Ethanolamine	**100.00**	**1.65**	33.34	3.34
$-NH_2$	$-CH_3$	-H	*R*-(-)-2-Amino-1-Propanol	107.91	2.77	109.94	15.42
$-NH_2$	$-CH_3$	-H	*S*-(+)-2-Amino-1-Propanol	93.75	4.02	22.38	1.87
$-NH_2$	$-(CH_3)_2$	-H	2-Amino-2-Methyl-1-Propanol	25.58	3.98	46.41	6.57
$-NH_2$	$-CH_2CH_3$	-H	*S*-(+)-2-Amino-1-Butanol	114.07	5.84	40.72	4.93
$-NH_2$	$-CH_2CH_3$	-H	*R*-(-)-2-Amino-1-Butanol	101.74	5.76	130.40	9.10
$-NH_2$	-COOH	-H	L-Serine	0.94	2.25	0.00	0.00
$-NH_2$	-H	$-CH_3$	*R* (-) 1-Amino-2-Propanol	52.41	4.50	7.16	0.60
$-NH_2$	-H	$-CH_3$	*S* (+) 1-Amino-2-Propanol	6.07	2.13	1.37	0.15
$-CH_2NH_2$	-H	-H	3-Amino-1-Propanol	37.52	2.96	44.52	4.33
$(CH_2)_2NH_2$	-H	-H	4-Amino-1-Butanol	0.05	1.93	59.95	6.00



(a) (b) See Table 2

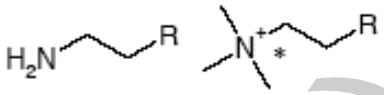
Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Table 4: Substrate specificity of *Tb*EK1 and *Tb*C/EK2 to utilise analogues modified at the hydroxyl group of the acceptor substrate.

R	*Compound*	*TbEK1 Relative rate*	*S.D.*	*TbC/EK2 Relative rate*	*S.D.*
-OH	*Choline	0.03	1.59	**100.00**	**7.97**
-OH	Ethanolamine	***100.00***	***4.84***	33.34	3.34
-SH	2-Amino ethanethiol	0.01	0.51	0.96	0.30
$-(OCH_3)_2$	Amino acetaldehyde dimethyl acetale	0.73	0.63	0.57	0.12
$-SO_3H$	Taurine	0.00	0.13	0.02	0.00
-H	Ethylamine	0.01	0.26	0.00	0.00
$-OSO_3H$	2-Aminoethyl hydrogen sulphate	0.54	2.31	0.00	0.00
-OOH	Glycine	0.35	0.72	0.85	0.11
$-OPO_3H_2$	Phosphoethanolamine	0.25	3.32	1.35	0.29
$-OCH_3$	2-Methoxy ethylamine	0.00	0.02	1.49	0.28
$-PO_3H_2$	2-Aminoethyl phosphonic acid	0.08	0.66	0.80	0.18



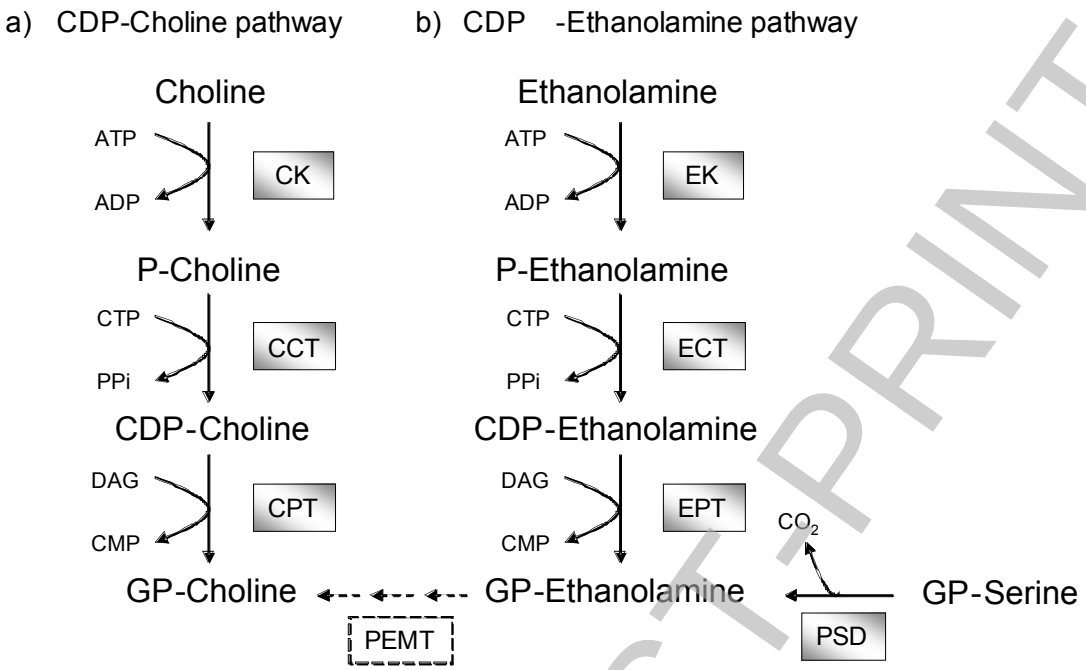
(a) (b) See Table 2

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 1

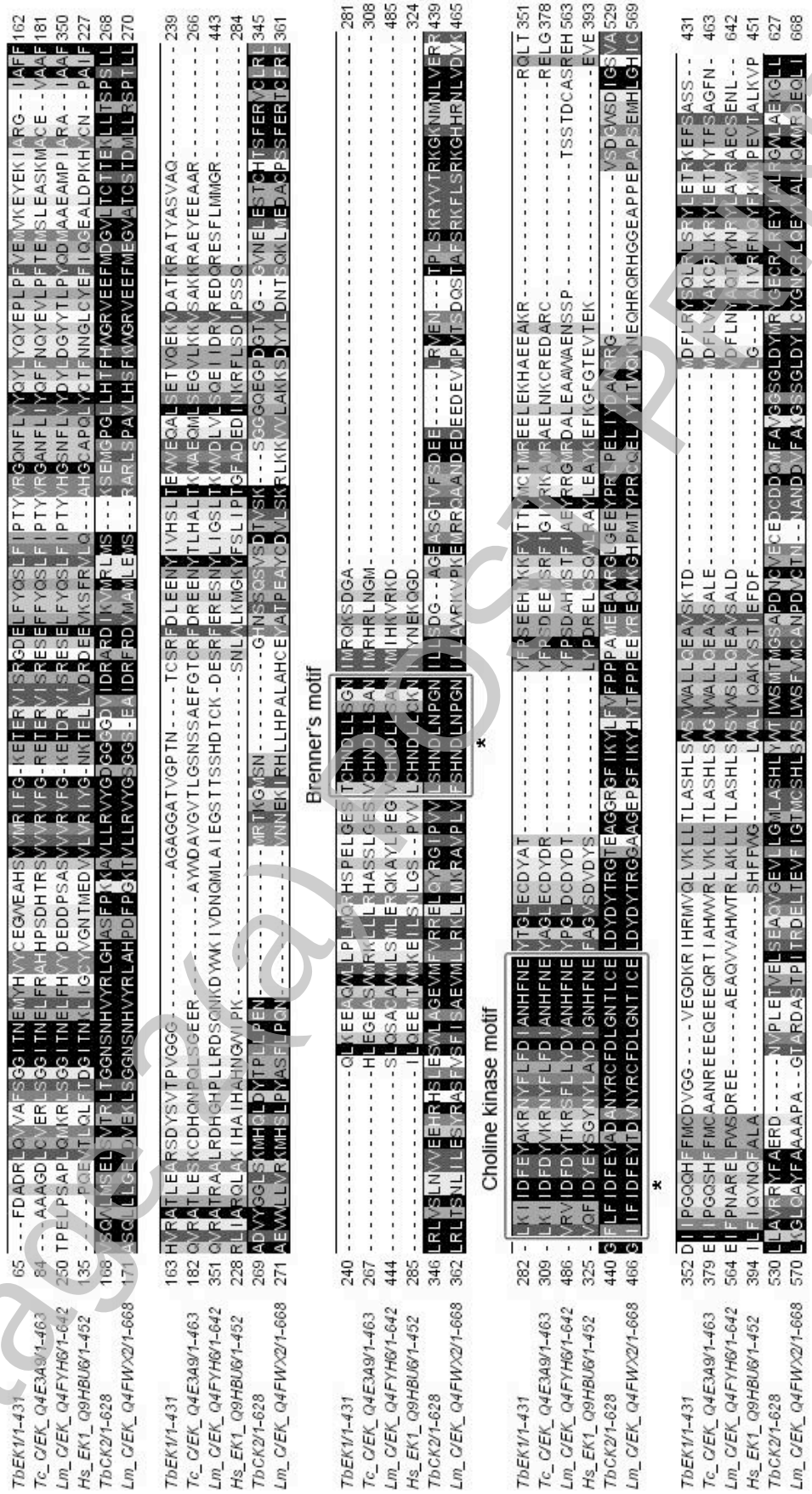


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 2

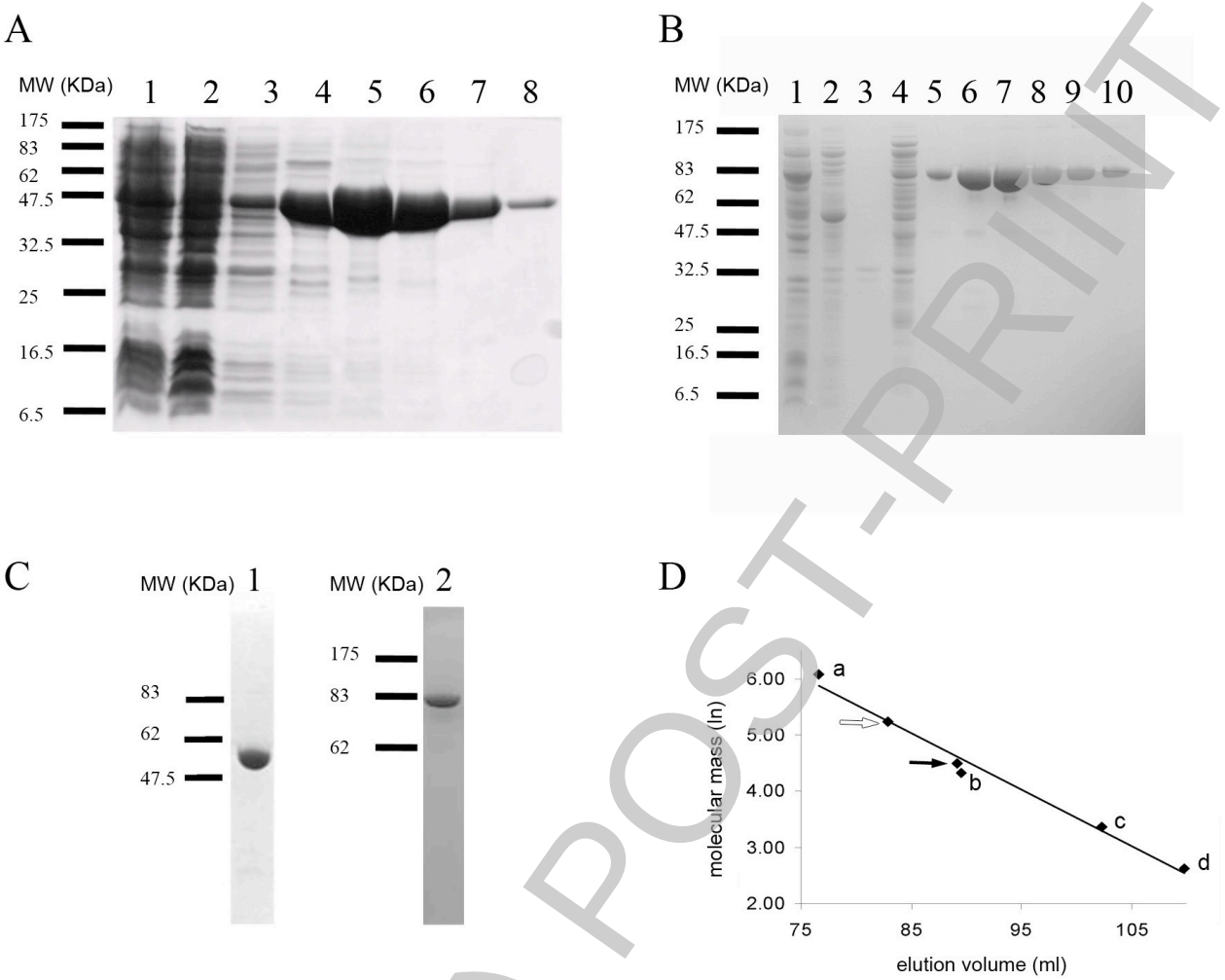


Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Stage 2(a) POSTPRINT

Figure 3

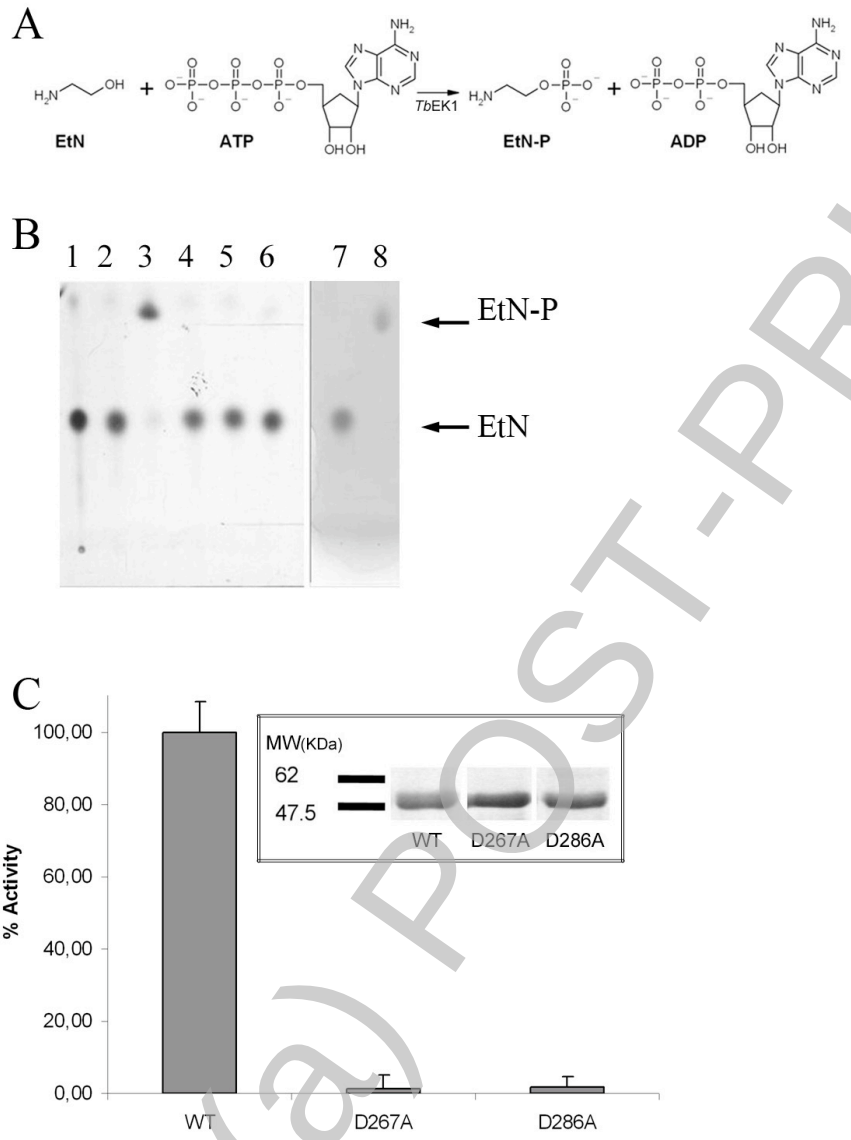


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 4

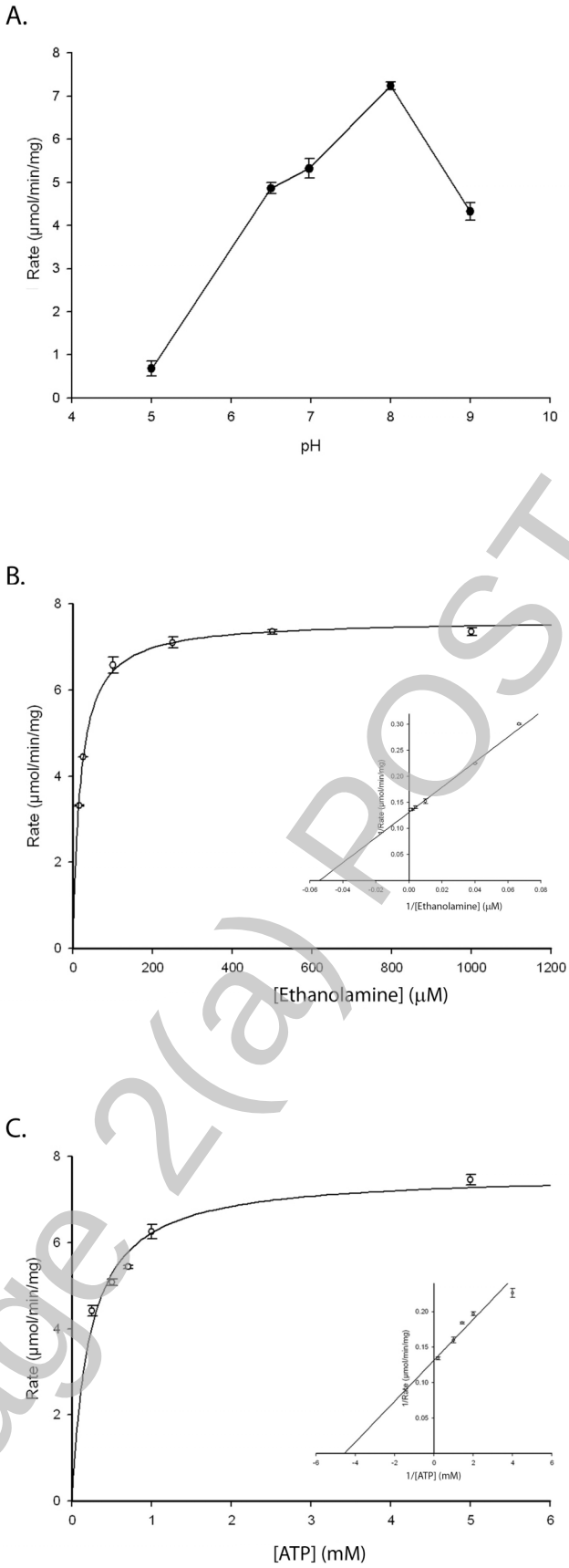


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 5

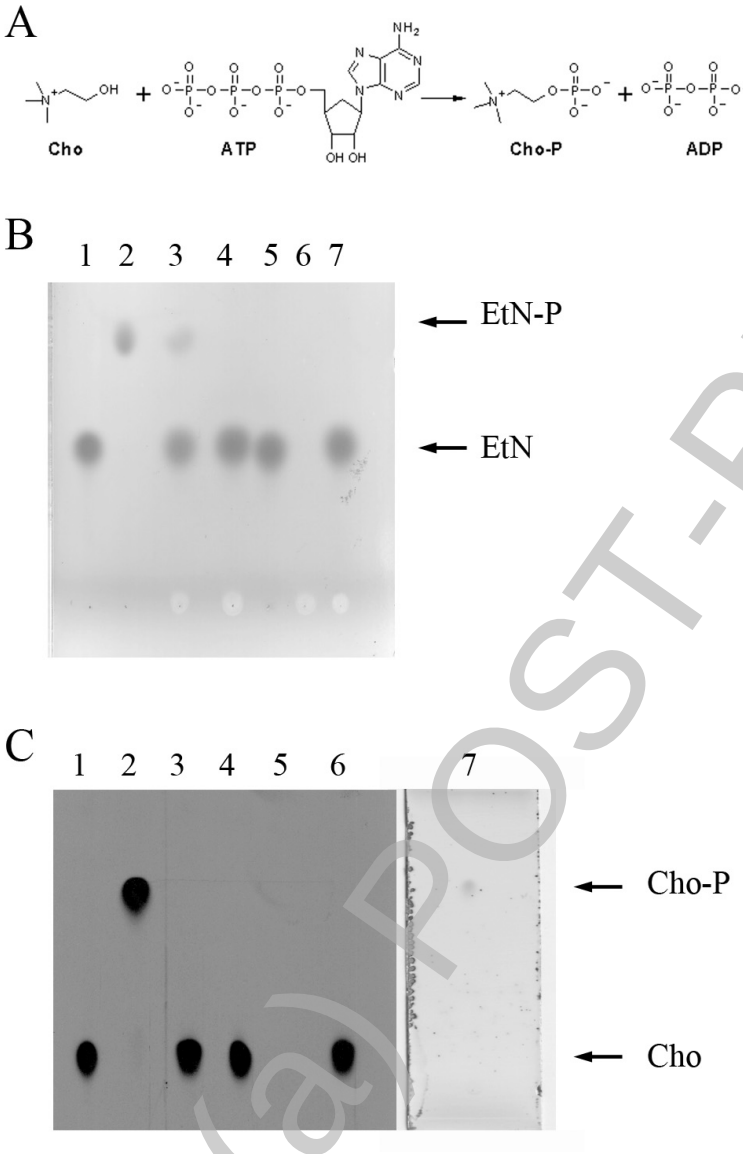


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 6

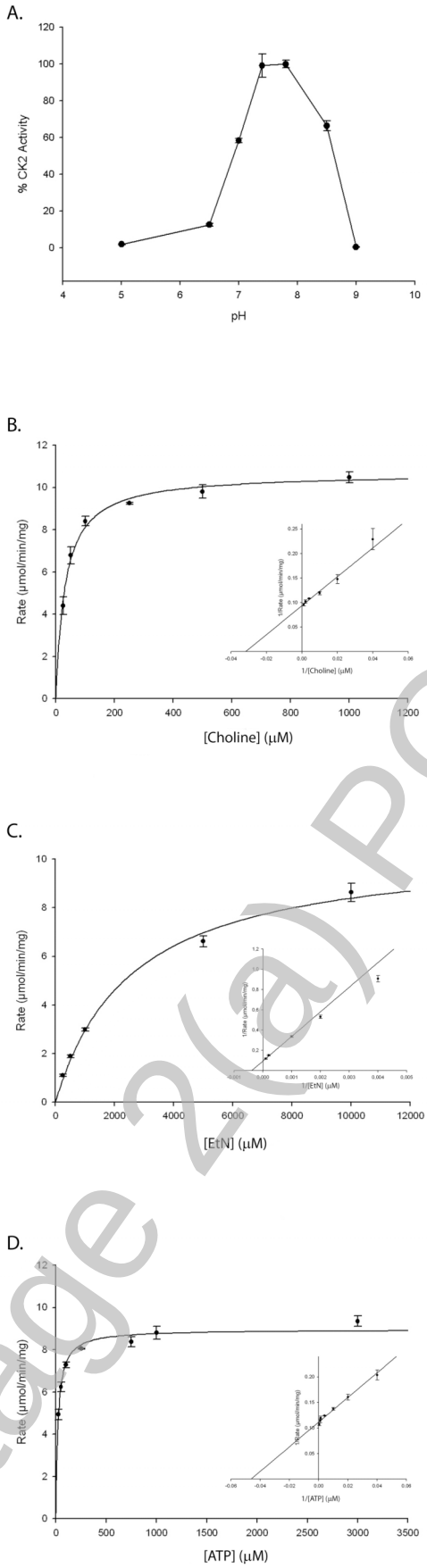


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 7



Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080435

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society