Mitochondrial energy metabolism and Krebs cycle activities are developmentally regulated in the life cycle of the protozoan parasite Trypanosoma brucei. Here we report cloning of a T. brucei aconitase gene that is closely related to mammalian iron-regulatory protein 1 (IRP-1) and plant aconitases. Kinetic analysis of purified recombinant TbACO expressed in Escherichia coli resulted in a $K_m$ (isocitrate) of $3 \pm 0.4 \text{ mM}$, similar to aconitases of other organisms. This was unexpected since an arginine conserved in the aconitase protein family and crucial for substrate positioning in the catalytic center and for activity of pig mitochondrial aconitase (Zheng, L., Kennedy, M. C., Beinert, H., and Zalkin, H. (1992) J. Biol. Chem. 267, 7895–7903) is substituted by leucine in the TbACO sequence. Expression of the 98-kDa TbACO was shown to be lowest in the slender bloodstream stage of the parasite, 8-fold elevated in the stumpy stage, and increased a further 4-fold in the procyclic stage. The differential expression of TbACO protein contrasted with only minor changes in TbACO mRNA, indicating translational or post-translational mechanisms of regulation. Whereas animal cells express two distinct compartmentalized aconitases, mitochondrial aconitase and cytoplasmic aconitase/IRP-1, TbACO accounts for total aconitase activity in trypanosomes. By cell fractionation and immunofluorescence microscopy, we show that native as well as a transfected epitope-tagged TbACO localizes in both the mitochondrial aconitase gene that is closely related to mammalian iron-regulatory protein 1 (IRP-1) and plant aconitases. Kinetic analysis of purified recombinant TbACO expressed in Escherichia coli resulted in a $K_m$ (isocitrate) of $3 \pm 0.4 \text{ mM}$, similar to aconitases of other organisms. This was unexpected since an arginine conserved in the aconitase protein family and crucial for substrate positioning in the catalytic center and for activity of pig mitochondrial aconitase (Zheng, L., Kennedy, M. C., Beinert, H., and Zalkin, H. (1992) J. Biol. Chem. 267, 7895–7903) is substituted by leucine in the TbACO sequence. Expression of the 98-kDa TbACO was shown to be lowest in the slender bloodstream stage of the parasite, 8-fold elevated in the stumpy stage, and increased a further 4-fold in the procyclic stage. The differential expression of TbACO protein contrasted with only minor changes in TbACO mRNA, indicating translational or post-translational mechanisms of regulation. Whereas animal cells express two distinct compartmentalized aconitases, mitochondrial aconitase and cytoplasmic aconitase/IRP-1, TbACO accounts for total aconitase activity in trypanosomes. By cell fractionation and immunofluorescence microscopy, we show that native as well as a transfected epitope-tagged TbACO localizes in both the mitochondrial and in the cytoplasm (70%). Together with phylogenetic reconstructions of the aconitase family, this suggests that animal IRPs have evolved from a multicompartmentalized ancient aconitase. The possible functions of a cytoplasmic aconitase in trypanosomes are discussed.

Trypanosoma brucei is a protozoan parasite in the blood and tissue fluids of mammals and causes two major tropical diseases, sleeping sickness in man and nagana in cattle. The flagellate is transmitted in subsaharan Africa between humans, livestock, and a huge reservoir of game animals by the tsetse fly, a bloodsucking insect vector. During cyclical transmission between mammals and tsetse, trypanosomes differentiate into a series of life cycle stages with distinct morphology, metabolism, and surface proteins to cope with the changing host environments and host defense mechanisms (1). The proliferating forms in the host blood have elongated and slender appearance (long slender forms) and rely entirely on glycolysis for energy production (2–4). Inefficient substrate utilization seems probable at that developmental stage, due to glucose homeostasis in host blood. Hence, slender forms lack significant amounts of Krebs cycle enzymes (5), and their residual mitochondrion does not contribute to ATP production. At the peak of a parasitemic wave, slender forms differentiate into a quiescent, cell cycle-arrested stage with stumpy morphology (stumpy form) which has a more developed mitochondrion and expresses citric acid cycle activities and an incomplete electron transport chain (5–8). In culture, differentiation to the stumpy stage is induced by a cell density sensing mechanism acting via the cAMP second messenger pathway (9, 10). A population of stumpy forms, in turn, can be triggered to differentiate rapidly and synchronously to the next stage in the life cycle, the procyclic forms, which populate the fly midgut and rely on respiration of proline as their major energy source. Therefore, induction of Krebs cycle enzymes and of a respiratory chain in stumpy forms is regarded as a preadaptation to the fly environment (8). The signal for differentiation to procyclic forms in the midgut of the tsetse is unknown; however, the process can be triggered in culture by a temperature shift to 27 °C or most efficiently by addition of cis-aconitate or citrate to the culture medium (11, 12). The differentiation inducing effect of cis-aconitate and citrate is highly specific. The peculiar role of these substrates in triggering a developmental switch and the coordinate induction of the citric acid cycle enzymes during differentiation to the stumpy stage prompted us to identify and characterize the aconitase gene of T. brucei.

Aconitase (citrat(isocitrat)hydro-lyase, EC 4.2.1.3) catalyzes the stereospecific dehydration/rehydration reaction of citrate to isocitrate via the intermediate cis-aconitate (13). Activity of the enzyme critically depends on the presence of an iron sulfur [4Fe4S] cluster in the catalytic center that is highly sensitive to oxygen. Upon oxidation the cluster is converted to an inactive [3Fe4S] form (14). Mammalian cells express two distinct aconitases encoded by separate genes: (a) mitochondrial aconitase and (b) a bifunctional cytoplasmic aconitase.

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A Developmentally Regulated Aconitase Related to Iron-regulatory Protein-1 Is Localized in the Cytoplasm and in the Mitochondrion of Trypanosoma brucei

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that is identical to the iron-regulatory protein (IRP-1). IRP-1 is a RNA-binding protein interacting with iron-responsive elements (IREs) in the untranslated regions of several mRNAs (15), including the transferrin receptor and ferritin H- and L-subunits (16), and functions in coordinate post-transcriptional regulation of cellular iron metabolism (17, 18). In iron-loaded cells, IRP-1 assembles a cubane [4Fe-4S] cluster, which renders it active as a cytosolic aconitase but inactive for RNA binding. Disassembly of the cluster upon iron starvation yields the active RNA-binding form that regulates stability or translation of target RNAs. The two conformations and functional states are mutually exclusive. Cytoplasmic aconitase activity might also be required for a glyoxalate cycle in specialized animal tissues like brown adipose tissue (19). In plants, where cytoplasmic aconitase is developmentally regulated and abundant in germinating seeds, its role in the glyoxalate cycle is well established (20, 21).

Here we report on cloning and characterization of a protozoan aconitase that is closely related to animal IRPs and plant aconitases. Dual subcellular localization of the developmental clone T. brucei enzyme suggests that it not only takes part in the mitochondrial Krebs cycle but may have a yet unknown function in the cytoplasm of the parasite.

**EXPERIMENTAL PROCEDURES**

Trypanosomes, Cell Culture, and Transfection—Bloodstream forms of the bloodstream T. brucei brucei clone AnTat1.1 were grown in rodents as described (22). The monomorphic clone MiTat1.4 (23) was digested with NcoI, nitrilotriacetic acid.

DNA was electroporated (BTX 600 Electro Cell Manipulator, 1.2 kV, 25 μF, 186 ohms, 2-mm electrode distance) into 4 × 10⁶ cells, 9 μl of transformation buffer, 4.5 μl of DNA, and 20 μl of fresh medium. After electroporation, the mixture of cells and medium was incubated at 45 °C, and 2 min 72 °C) using a XhoI/SalI/UClA d(T)₁₇ primer (5’ GGACTGGATCGTCACTATGAT₃’), followed by PCR with the adaptor primer 5’ GATCGTTCGAGCATCG 3’ and the TAICO-specific nested primers 5’ ATGGCCAAGAATCCATA-CATA 3’ (RIRP-2) and 5’ TGGCAACACCGCAGTTC 3’ (RIRP-1) or with adaptor primer and primer 5’ GGGAATCTGTTGATAGG 3’ (IRP-6).

DNA Sequencing—The sequence of the antisense strand was determined from overlapping deletion clones of plasmid Tbaco1.1, constructed as recommended by the supplier of the Nested Deletion Kit (Amersham Pharmacia Biotech). The nucleotide sequences of the sense strand, of DNA clones, and of the genomic clone obtained by inverted PCR, are deposited in the GenBank database. The Sequenew™ version 2.0 Sequencing Kit (U. S. Biological Chemical Corp.) and fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems) were used for manual and automated sequencing, respectively.

Plasmid Constructs and Site-directed Mutagenesis—The complete coding region of TAICO was assembled from a 104-bp Psf1-EcoRV fragment derived from a 3’-RACE clone and the genomic clone Tbaco1.1 opened with Psf1 (partial digest) and XbaI (blunted). For convenient cloning, a TAICO cassette was constructed as follows: a BamHI site was introduced immediately upstream of the start codon by recombinant PCR using the primers 5’ CGGGATCATGATCCAGACGAT-AA 3’ and JS5-2 (see cDNA cloning), and a BglII site was introduced exactly after the stop codon with the primers RIRP-2 (see cDNA cloning) and 5’ GAGAATCTTACAAAATTACCCGATT 3’ (IRP-3). The cassette was then recloned between BamHI and SalI of the insertion vector pLew20 which targets the rDNA spacer of T. brucei. Details of these constructions are available upon request.

For bacterial expression, the following TAICO fragments were cloned into the hexahistidine tag expression vector pQE30 (Qiagen): a StuI-PsiI fragment of Tbaco1.1 resulting in pQe31/13 (88 kDa), a BamHI-PsiI fragment of Tbaco1.1 resulting in pQe33/4 (80 kDa), and the full-length TAICO cassette excised with BamHI and SalI resulting in pQe35/5 (99 kDa). A Cys-446 for high stringency conditions and to 40 °C below the calculated melting 1° C.

**3 The abbreviations used are: IRP, iron-regulatory protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IRE, iron-responsive element; kb, kibibase; Me2S, 2-(N-morpholino)-ethanesulfonic acid; NBT, nitro blue tetrazolium; PFC, procaryotic forms; PCR, polymerase chain reaction; SS, short stumpy forms; UTR, untranslated region; bp, base pair; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; NTA, nitrotriacetic acid.
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DINITRIUM ISOThIOcyanate/CSCL2 CUSHION METHOD (32) AND WAS FRACTIONATED FROM T. BRUCELLI FRAGMENT. 99702.7 (ALL PERFORMED BY THE PROTEIN CHEMISTRY CORE FACILITY OF THE MPI FOR MOLLUSCA). THE PREDICTED SEQUENCE OF RECOMBINANT TBACO, AND A MASS SPECTRUM OBTAINED BY MICROSEQUENCING; AN AMINO ACID ANALYSIS GAVE RESULTS COMPATIBLE WITH THE TRANSLATION OF THE PREVIOUS RT-PCR PRODUCTS. CHROMATOGRAPHY ON A MONOQ HR 5/5 COLUMN (AMERSHAM PHARMACIA LKB) WAS USED TO FURTHER FRACTIONATE THE PREVIOUS PRODUCTS. THE YIELD WAS IN THE RANGE OF 230 TO 600 OF 0.7, AND TBACO EXPRESSION MESSAGES (WITHOUT 3'-UTR. THE 3'-ENDS OF TBACO TRANSCRIPTS (7) AS WELL AS 254 BP OF 3'-UTR. THE TRANS-SPlice SITE (MARKED BY AN A BULGE) WAS DETECTED AT LOW HYBRIDIZATION STRINGENCY (40 °C BELOW THE CALCIUM-PROTEIN CUSHION METHOD). FOR THE UV ASSAY DESCRIBED BY HENSON AND CLELAND (37), 1 ML OF 90 mM TRIS-HCL, pH 7.5, CONTAINING dISOcITRATE (SIGMA) RANGING FROM 1 TO 20 mM WAS EQUILIBRATED AT 25 °C, AND THE FORMATION OF cis-Aconitate WAS DETECTED IN MINUTES (EXTINCTION COEFFICIENT FOR cis-Aconitate ε = 3.4 × 10⁴ M⁻¹ cm⁻¹ AT 240 nm). ASSUMING MICHAELS-MENTEN KINETICS, NONLINEAR CURVE FITTING OF KINETIC DATA WAS PERFORMED WITH KALEDISGRAPh 3.0.8 (SYNERGY SOFTWARE) AND THE Kₚ VALUES WERE CALCULATED FROM A RECIPROCAL PLOT ACCORDING TO HANES (38). ACONITASE ACTIVITY IN WHOLE CELL EXTRACTS WAS MEASURED AS DESCRIBED BY OVERARTH ET AL. (7). PROTEIN CONCENTRATIONS WERE DETERMINED USING THE BRADFORD ASSAY AS SUPPLIED BY BIO-RAD AND BOVINE SERUM ALBUMIN AS STANDARD.

ANTIBOLES—FOR THE FIRST SERIES OF IMMUNIZATIONS (INCLUDING "RAT 1"), AN 88-KDA RECOMBINANT TBACO FRAGMENT (pQE31/13) WAS PURIFIED BY Ni²⁺-NTA CHROMATOGRAPHY, ELECTROPHORESED ON AN 8% (w/v) SDS-POLYACRYLAMIDE GEL, AND BLOTTED ONTO NICOTINOXOLOCULLASE. THE 88-KDA BAND WAS EXCISED FROM THE MEMBRANE (150–200 ΟΟ OF PROTEIN) AND THE dINTACT DRY NICOTINOXOLOCULLASE WAS POWDERED, EMULSIFIED WITH COMPLETE FREUND ADJUVANT, AND INJECTED INTRAPERITONEALLY IN FEMALES OF WISTAR RATS. RATS WERE BOOSTERED ONCE WITH 150 ΟΟ OF PROTEIN AFTER 4 WEEKS. TWO WEEKS LATER, THE ANIMALS WERE BLED, AND SERA WERE AFFINITY PURIFIED AS DESCRIBED (39). FOR A SECOND SERIES OF IMMUNIZATIONS (INCLUDING "RAT 2" AND "RAT 1") THE RECOMBINANT FULL-LENGTH TBACO (pQE30/5/5) WAS PURIFIED BY Ni²⁺-NTA CHROMATOGRAPHY IN SOLUBLE FORM AND DIRECTLY EMULSIFIED WITH COMPLETE FREUND ADJUVANT. RABBITS WERE IMMUNIZED BY SUBDERMAL INJECTION OF 500 ΟΟ OF PROTEIN FOLLOWED BY 3 BOOSTER INJECTIONS AT REGULAR INTERVALS. RABBITS ANTI-PHOSPHOGLYCERATE KINASE C ANTIBODIES WERE A KIND GIFT OF P. MICHELS. RABBIT ANTI-SYNCHOCoccus sp. HSP60 ANTISERUM WAS PURCHASED FROM STRESSGEN BIOTECHNOLOGIES CORP. THE BB2 HYBRIDOMA (anti-Ty1 monoclonal antibody) WAS A KIND GIFT OF P. BASTIN AND K. GULL. RABBIT ANTI-DICTYOSTELIUM α-TUBULIN ANTISERUM WAS PURCHASED FROM GENETECH LKB.

IMMUNOBLOT ANALYSIS—WHOLE CELL EXTRACTS WERE PREPARED BY TRANSFERRING SMALL VOLUMES OF PBS-WASHED AND RESUSPENDED TRYPOSMONES INTO SDS-LYSIS BUFFER PREHEATED TO 100 °C. SAMPLES WERE BOILED FOR 5 MIN AND SONIFIED. PROTEINS WERE SEPARATED ON 8% (w/v) SDS-POLYACRYLAMIDE GELS AND WERE TRANSFERRED ONTO HYBON™ PDVF DIFLUORIDE MEMBRANES (AMERSHAM PHARMACIA BIOTECH) USING A SEMI-DRY BLOTTER.
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**Fig. 3.** Multiple sequence alignment of deduced TbACO amino acid sequence with representative aconitase family members. *A. thaliana* aconitase (78), GenBank accession number X82839, human IRP-1 (108), GenBank accession numbers M58510 and M37835, and porcine mitochondrial aconitase (109), GenBank accession number J05224 are aligned with TbACO. The numbering of Rutgers University Protein Database (110) entry 7ACN was adopted for pig heart aconitase. The alignment was compiled by ClustalW, version 1.60 (43), with default settings (gap opening penalty of 10 and gap extension penalty of 0.05). The presequence of porcine mitochondrial aconitase was not included, and manual adjustments of the alignment were made at the N terminus. Sequence identity with TbACO is indicated by black lettering. Additional residues highly conserved in the FeS isomerase family (53) are marked by an asterisk. The motifs initially chosen for design of degenerate PCR primers (see “Experimental Procedures”) are underlined.

**Cloning of an Aconitase Gene of *T. brucei***—Highly degenerate pools of PCR primers matching two conserved sequence motifs of the aconitase gene family were used to amplify genomic *T. brucei* DNA. Ten out of 31 clones derived from a PCR product of expected size contained the same 311-bp insert with an uninterrupted open reading frame exhibiting 74% protein sequence similarity to human IRP-1 and 75% similarity to *Arabidopsis thaliana* aconitase. No other aconitase family-related sequence was detected with this and other pairs of degenerate primers. The cloned 311-bp fragment was used to probe Southern blots of genomic DNA. Each restriction enzyme digestion procedured with ClustalW, version 1.60 (43), and manual editing. The total length of the alignment comprises 1171 sites and is available upon request. To reconstruct phylogenetic trees based on the amino acid sequence alignment, the PUZZLE program (version 4.0) was applied (46). As substitution model the substitution matrix BLOSUM 62 (44) was assumed. To account for rate heterogeneity a discrete Gamma model (47) with four categories was introduced, and the corresponding shape parameter was estimated. For each sequence we tested if the base composition deviates significantly from the average composition. The estimated shape parameter of the gamma distribution equals 1.17, thus indicating weak rate heterogeneity.

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SacII-PstI fragment was cloned (plasmid Thaco1.1, see “Experimental Procedures” and Fig. 2) which contained a long open reading frame devoid of a stop codon. Therefore, a cDNA containing the missing C terminus was cloned by reverse transcriptase-PCR with nested TbACO-specific primers. An 800-bp internally primed cDNA was obtained. Therefore, the 3′-end of the gene was cloned by inverted PCR (see Fig. 2 and “Experimental Procedures”). The length of the 5′-UTR of the TbACO transcript was determined by a modified 5′-RACE strategy exploiting the conserved mini-exon sequence present at the 5′-end of all trypanosomal mRNAs. The sequence of three independent clones derived from the single PCR product showed that the mini-exon was added at a trans-splice site (AG dinucleotide) at position −100 relative to the first ATG codon. As expected for a canonical trans-splice site, several polyadenylidine stretches were found within 50 bp upstream of the AG.

The TbACO Protein Sequence—The open reading frame predicted a protein of 897 amino acids with a calculated molecular mass of 98,302 Da and a pi of 6.48. Both methionines at positions 1 and 5 are in sequence environments compatible with the requirements for translation initiation (48). Thus, use of the first ATG is assumed. Pairwise alignments of TbACO with other members of the aconitate family show a very high degree of amino acid identity and similarity with vertebrate IRPs and with plant aconitases (58/74% with human IRP-1; 61/75% with A. thaliana aconitase) extending over the entire length of the proteins. In contrast, mitochondrial aconitases of mammals or S. cerevisiae are significantly more distant (30% identity, 55% similarity). A multiple sequence alignment of TbACO with a representative member of each aconitase subfamily is shown in Fig. 3. From the crystal structure and mutation analysis of porcine mitochondrial aconitase, 24 residues were identified as important for coordination of the [4Fe-4S] cluster, for substrate recognition and catalysis, and for support of active site chains by hydrogen bonds (49–52). Table I lists the homologous positions in TbACO, showing that 21 of 24 positions are conserved. Asparagine 170 and alanine 74 of pig mitochondrial aconitase have been implicated in hydrogen bonding with other active site residues but are not conserved in the Fe-S isomerase family (53). In TbACO these positions are substituted by methionine (Met-222) and phenylalanine (Phe-100), respectively, like in mammalian IRPs and plant aconitases (see Fig. 3). Arginine 580 that appears to be a key residue for substrate binding in pig mitochondrial aconitase (52) has been replaced by leucine (Leu-702) in TbACO. This position is only substituted in two other aconitase sequences, that of L. pneumophila and a hypothetical aconitase of S. cerevisiae (HACO) identified by genome sequencing (55). A multiple alignment of Frishman and Hentze (53) has identified 26 additional residues that are highly conserved in the Fe-S isomerase family (marked with an asterisk in Fig. 3). Of these, only one glycine (Gly-413 of pig mitochondrial aconitase) is conserved in all sequences. The high degree of sequence similarity of TbACO with IRPs, and the fact that trypanosomes represent the earliest branch of extant eukaryotes that harbor mitochondria (56), solicited a phylogenetic analysis. Sequences of 22 aconitases and IRPs available in data bases were aligned. As outgroup, sequences served 10 isopropylmalate isomerase and bacterial aconitase B group sequences that are significantly different. A PUZZLE tree based on 32 aligned sequences was reconstructed. For the 22 aconitase and IRP sequences a PUZZLE tree was computed and rooted according to the big tree (Fig. 4). The general topology of the tree is very similar to published trees of the aconitase family (53, 57, 58). TbACO is clearly separated from the mitochondrial aconitases. Within the IRP-like subfamily, TbACO, plant aconitases, bacterial aconitases, and IRPs all branch from one node, and it is not possible to resolve the branching pattern any further. The statistical support for any particular topology linking TbACO to either the IRP cluster or the plant aconitase cluster is weak. This is congruent with recent evidence for an overestimation of the divergence of protists and crown eukaryotes, based on nuclear gene data, and the proposal of a “big bang” radiation of the various eukaryotic lineages (reviewed in Ref. 59).

Enzymatic Activity and Kinetic Properties of Recombinant TbACO—The complete open reading frame of TbACO was fused to a hexahistidine tag at the N terminus, and the fusion protein was expressed in E. coli and purified by affinity chromatography on a metal chelate resin (Fig. 5A). Aconitase activity increased linearly with the amount of recombinant protein and was maximal between pH 7 and 8 (not shown). The activity was dependent on loading of the protein with Fe2+ in the presence of DTT and was sensitive to oxygen (Table II), consistent with the presence of a labile [4Fe-4S] cluster (14, 36, 60). The conserved cysteine positions of TbACO ligating this putative iron-sulfur cluster (see Table I) were then substituted by serines by site-directed mutagenesis. Two recombinant mutant proteins (C446S and C512S/C515S) were purified and iron-loaded exactly as the wild type protein. As expected, no enzymatic activity could be detected (Table II and data not shown). The analogous mutations in human IRP-1 also abolished enzymatic activity (61, 62). This control provided unambiguous evidence that we had cloned an aconitase. The specific activity under V max conditions was determined using >95% pure recombinant enzyme prepared by further purification on a MonoQ™ ion exchange column (Fig. 5A). The result was close to specific activities reported for bovine heart mitochondrial...
aconitase (30 μmol min⁻¹ mg⁻¹ (36, 52)), bovine IRP (34 μmol min⁻¹ mg⁻¹ (63)), and potato tuber mitochondrial aconitase (32 μmol min⁻¹ mg⁻¹ (64)). The Michaelis-Menten constant (K_m) for the substrate isocitrate was determined from reaction velocity plots and derived reciprocal Hanes plots as shown in Fig. 5B. The resulting K_m of 3 ± 0.4 mM is about 1 order of magnitude higher than published values for pig heart mitochondrial aconitase (65). This can be correlated with the substitution of an active site residue implicated in substrate binding (Leu-702) in T. brucei mitochondrial aconitase (66). This can be correlated with the substitution of an active site residue implicated in substrate binding (Leu-702) in T. brucei mitochondrial aconitase (66). This can be correlated with the substitution of an active site residue implicated in substrate binding (Leu-702) in T. brucei mitochondrial aconitase (66). This can be correlated with the substitution of an active site residue implicated in substrate binding (Leu-702) in T. brucei mitochondrial aconitase (66). This can be correlated with the substitution of an active site residue implicated in substrate binding (Leu-702) in T. brucei mitochondrial aconitase (66).

**FIG. 4. Phylogenetic tree of the aconitase family.** A PUZZLE tree was calculated for an alignment of 22 aconitases and IRPs (see "Experimental Procedures"). The tree was rooted with the information from a second tree that included 10 outgroup sequences (isopropylmalate isomerases and bacterial aconitase B group). The numbers along the edges indicate the support values (% of trees in a sample of 10,000 trees that show the same grouping (44)). The branch length is a measure of the number of substitutions (see scale). All sequences used for the alignment are available in public data bases. Accession numbers are listed in Refs. 55, 58, and 73.

**Fig. 5. Kinetic analysis of recombinant TbACO.** Hexahistidine-tagged TbACO was expressed in E. coli and purified as described under “Experimental Procedures.” A. Coomassie Brilliant Blue-stained (1st to 3rd lanes) or silver-stained (4th lane) 8% (w/v) SDS-PAGE; 1st lane, bacterial lysate of the transformed E. coli M15rep4 (-IPTG); 2nd lane, bacterial lysate of the transformed E. coli M15rep4 (+IPTG); 3rd lane, protein eluted from a nickel chelate affinity column (Ni²⁺-NTA); 4th lane, protein eluted from a second step MonoQ HR 5/5 ion exchange resin (MonoQ). Most of the faster migrating bands detected by silver staining (4th lane) are degradation products as indicated by immunoreactivity with anti-TbACO antibodies (not shown). TbACO eluted from Ni²⁺-NTA was activated with Fe²⁺ and DTT under nitrogen atmosphere. Kinetic measurements with activated protein were performed with isocitrate as substrate, and formation of cis-aconitate was monitored spectroscopically at 240 nm. B, data representation as reciprocal [S]/[v] plot according to Hanes (38) with the original [v]/[S] (data concentration; v, reaction velocity) plot as inset. Dots indicate mean values, and error bars indicate standard deviations of a triplicate measurement. K_m = 2.9 mM and V_max = 10.6 μmol min⁻¹ mg⁻¹ were determined from the experiment shown. A mean of K_m = 3 ± 0.4 mM for the substrate isocitrate was calculated from five measurements with three independent protein preparations.
Trypanosome Aconitase

Table II

Enzymatic activity of recombinant purified TbACO

| Enzyme | Activation | Anaerobic conditions | Reaction | Specific activity | µmol min⁻¹ (mg protein)⁻¹ |
|--------|------------|----------------------|----------|------------------|--------------------------|
| rTbACO | Fe²⁺/DTT   | –                    | Isocitrate to cis-aconitate | Not detectable |
| rTbACO | +          | –                    | Isocitrate to cis-aconitate | 2, variable |
| rTbACO | +          | +                    | Isocitrate to cis-aconitate | 10.6 |
| C512SC5158' | + | + | Isocitrate to cis-aconitate | Not detectable |
| rTbACO/MonoQ™ | + | + | Isocitrate to cis-aconitate | 15 ± 1.1 |
| rTbACO/MonoQ™ | + | + | Citrate to cis-aconitate | 8.3 ± 0.23 |

a Expressed in E. coli and purified by affinity chromatography on Ni²⁺-NTA as described under “Experimental Procedures.”

b Further purification by ion exchange chromatography on MonoQ™ as described under “Experimental Procedures.”

c Calculated from Fig. 5B.

d Mean ± S.D. of triplicate enzyme assay at 25 mM isocitrate.

Fig. 6. Developmental profile of TbACO mRNA expression. A Northern blot with 5 µg of total RNA isolated from a long slender (LS) bloodstream form population, a short stumpy (SS) bloodstream form population, and cultured procyclic forms (PCF) of strain Antat1.1 was probed with a riboprobe derived from the coding region of TbACO. In the SS population, 90% of the cells expressed the stumpy cell marker NADH dehydrogenase, as assayed by cytochemical staining (6). Equal the SS population, 90% of the cells expressed the stumpy cell marker TbACO. Based on different choles-

terior content of the membranes, only the plasma membrane is permeabilized at low digitonin concentrations, whereas org-

nellar membranes require higher concentrations (68–70). Phosphoglcyerase kinase B and the mitochondrial heat shock protein HSP60 were used as markers for the cytosolic and mitochondrial fractions, respectively, as described before (41). Procytic cells were incubated with increasing concentrations of digitonin, followed by centrifugal separation of soluble and particulate fractions. The amount of TbACO and of the marker proteins in each fraction was quantified by densitometric scan-

ning of Western blots (Fig. 8, A and B). About 70% of total TbACO was released into the supernatant together with the cytosolic marker phosphoglyceraldehyde kinase B at 0.5 mg of digiton per mg of cellular protein. The remaining part of TbACO appeared to be as resistant to solubilization as the mitochondrial matrix marker HSP60. To exclude the possibility that our antibodies cross-reacted with a different 98-kDa protein in one of the cellular compartments, TbACO was epoite-tagged at the C terminus with a short peptide derived from the yeast transposable element Ty1 (29). The tagged open reading frame was inserted into the trypanosomal expression vector pLew20 (28) and targeted to the ribosomal spacer region of the T. brucei genome by homologous recombination. The resulting procyclic cell line, which expressed about the same amount of tagged TbACO as wild type protein (not shown), was subjected to digitonin fractionation using the same conditions as for wild type procyclic forms. Western blots were probed with an epoite tag-specific mononclonal antibody (Fig. 8, C and D). About 70% of the tagged TbACO was released together with the cytosolic marker (phosphoglyceraldehyde kinase B) at low digitonin concentrations where the mitochondrial HSP60 was quantita-

tively retained in the pellet fraction. Together, these experi-

ments proved that about 70% of TbACO was localized in the cytoplasm of procyclic T. brucei. Mitochondrial localization of the remaining 30% was suggested by the fractionation; how-

ever, the protease digestion control required to rule out non-

pecific association of TbACO with the particulate fraction was technically difficult due to relative resistance of free TbACO to digestion. Instead, localization of TbACO to the single mito-

ochondrion of trypanosomes was directly documented by immu-

nofluorescence microscopy. In the stumpy stage, the mitochon-

drion has an easily discernible tubular structure extending over the entire length of the cell and can be visualized with the aldehyde-fixable membrane potential sensitive fluorescent dye MitoTracker™ Green FM (42). Upon immunofluorescent staining with three different TbACO-specific antibodies, perfect colocalization of the antibody signal with the MitoTracker mitochondrial marker was observed in every cell (Fig. 9 and not shown). Double immunofluorescent staining with TbACO antibodies and antibodies detecting the mitochondrial matrix protein HSP60 also showed colocalization and confirmed antibody...
access to the mitochondrial matrix under the given permeabilization conditions (data not shown). TbACO-specific cytoplasmic staining was weak, as expected from leakage of cytoplasmic protein during permeabilization necessary for detection of mitochondrial proteins (Fig. 9). In conclusion, TbACO has a dual subcellular localization in the cytosol and in the mitochondrion of T. brucei. Cytosolic and mitochondrial aconitase activity was then measured in lysates after preparative fractionation of procyclic trypanosomes with 0.5 mg of digitonin per mg of total protein. The fractionation was controlled with the marker proteins phosphoglycerate kinase B and HSP60, and the enzymatic assay showed that 78 ± 9% of the activity was in the cytoplasmic fraction and 21 ± 2% was associated with organelles. Thus, the subcellular distribution of aconitase activity was in agreement with the distribution of TbACO protein.

DISCUSSION

Mammalian cells have two aconitases encoded by separate nuclear genes: (a) the mitochondrial citric acid cycle enzyme and (b) a cytoplasmic aconitate, better known as the iron-regulatory protein (IRP-1) which acts as an iron sensor and post-transcriptional regulator and as a signal transducer for oxidative stress (71, 72). Since members of both aconitase subfamilies were identified in invertebrates, e.g. Caenorhabditis elegans and Drosophila melanogaster (73), specialization of mitochondrial and cytoplasmic aconitase functions seems to have occurred early during animal evolution. Here we report on a protozoan aconitate which belongs to the IRP-1 subfamily but localizes in the mitochondrion as well as in the cytoplasm. TbACO accounts for total aconitase activity in T. brucei. This was directly confirmed by enzyme assays in trypanosome lines with targeted disruption of both alleles of TbACO. In several independent Δaco::HYG/Δaco::NEO procyclic lines,2 no aconitase activity could be detected (data not shown). The sensitivity of the enzymatic assay does not exclude a minor activity; however, three independent methods, PCR amplification with degenerate primers, low stringency Southern hybridizations, and Western blotting with several TbACO antisera (Fig. 1 and Fig. 7, and not shown), did not indicate a second aconitase or a second IRP-related gene in T. brucei.

The mitochondrial localization of about 30% of TbACO and the absence of a second aconitase indicate that TbACO functions in the citric acid cycle of T. brucei. To our knowledge, this is the first direct evidence that an IRP-like aconitate functions in mitochondrial metabolism in a eukaryote. The dual subcellular localization resembles the situation in plant tissues, where 50–90% of total aconitase activity or protein reside in the cytosol and function in the glyoxalate cycle (20, 21, 74, 75). Plant aconitate purified from cytosol and mitochondria is indistinguishable with respect to kinetic parameters, molecular mass (90–98 kDa), and the EPR spectrum (20, 64, 74–76). Furthermore, the molecular mass and the EPR spectrum of plant aconitate purified from mitochondria resemble mammalian IRP but not mammalian mitochondrial aconitase (64, 77). The three plant aconitate sequences available so far (21, 78) are very similar to IRPs and to TbACO but less related to the mitochondrial aconitase subfamily. Hence, it seems likely that the mitochondrial aconitate in plants is encoded by these IRP-related sequences, although direct evidence has yet to be provided. Particularly, it is unknown whether the cytosolic and mitochondrial plant aconitate isoforms, which are differentially regulated and chromatographically separable (20), derive from a single or distinct genes. The dual localization of the T. brucei aconitate, which is similar to plant aconitases (Fig. 3), suggests that cytosolic and mitochondrial IRP-related isoforms may indeed be encoded by one gene in plants.

As the first IRP-like sequence from a protist, the TbACO sequence has added valuable phylogenetic information with respect to evolution of the aconitase gene family (53, 57, 58, 79). The fact that TbACO forms a well supported clade together

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2 B. Fast and M. Boshart, unpublished data.
with animal IRPs, plant aconitases, and bacterial aconitases of the *E. coli* *acoA* type, clearly separated from mitochondrial aconitases of yeast and animals (Fig. 4), strengthens the argument for the existence of aconitase paralogues before the separation of eubacteria from eukaryotes. The mitochondrial localization of TbACO and the branching of IRP, TbACO, and bacterial aconitases from one node support the view that the ancestor of the regulatory protein IRP was an enzyme of mitochondrial energy metabolism which most likely was acquired from a proteobacterial endosymbiont. It seems that members of the IRP-like and of the classical mitochondrial aconitase subfamilies both can function in more than one subcellular compartment. In *S. cerevisiae*, which does not harbor an IRP-related gene in its genome (80), the mitochondrial aconitase is also present in the cytosol where it participates in the glyoxalate cycle (81). In trypanosomes, the single IRP-like aconitase is present in the mitochondrion and in the cytosol, and in plants, IRP-related aconitases seem to function in mitochondrial metabolism as well as in the cytosolic step of the glyoxalate cycle (20, 21, 75). Animals may have kept or acquired members of both aconitase subfamilies during evolution and restricted them to one subcellular compartment in order to specialize them for different tasks in the mitochondrion and in the cytosol.

The distribution of one gene product between different subcellular compartments can be achieved by a variety of mechanisms, most of which lead to alternative transcription and/or translation initiation sites or alternative mRNA processing (reviewed in Ref. 82). In the case of TbACO, there is only one defined 5’-end of the mRNA and two closely spaced potential AUG codons at positions 1 and 5. It is difficult to imagine alternative translation initiation at these positions to include a mitochondrial import presequence in a fraction of the synthesized protein. Furthermore, the N-terminal sequence of TbACO does not form an amphipathic helix and does not predict a classical mitochondrial targeting signal. The mechanisms for protein import in trypanosomes seem to be largely conserved (41, 83–86); however, a second class of much shorter, typically 9 amino acid long presequences that resemble hydrogenosomal protein import sequences has been identified (87). At least one imported protein (cytochrome *c₁*) lacks a presequence (88), and a nonconservative import pathway has been suggested (86). Mitochondrial and cytoplasmic TbACO were never resolved as double band upon PAGE of *T. brucei* lysates, and hence, there is no evidence for cleavage of a leader sequence. However, cleavage of both mitochondrial and cytosolic mature protein as shown for *S. cerevisiae* fumarase FUM1 (89) cannot be excluded. All fumarase molecules synthesized in yeast are processed by the mitochondrial matrix protease, but nevertheless most of the enzyme (80–90%) ends up in the cytosol. An aborted translocation process has been suggested to be responsible for the dual mitochondrial and cytoplasmic localization of *S. cerevisiae* fumarase FUM1 (89). It should be noted that the 9 amino acid presequence of *T. brucei* dihydrolipoamide dehydrogenase which is sufficient for import (85, 87) is cleaved off in both bloodstream and procyclic forms, although the protein is not present in the mitochondrion of slender bloodstream forms (90–93). We suggest that multicompartmentalization of TbACO is due to inefficient targeting or an aborted translocation mechanism, possibly associated with a weak import signal.

**Fig. 8. Subcellular localization (digitonin fractionation).** Procyclic trypanosomes were subjected to differential permeabilization by 0–10 mg of digitonin/mg of cellular protein (concentration indicated on top of the lanes in A and C, a different batch of digitonin was used for C) and were subsequently fractionated by centrifugation. Supernatant (*S*) and pellet (*P*) fractions were run on 8 or 10% (w/v) SDS-polyacrylamide gels and were analyzed by Western blotting using anti-phosphoglycerate kinase C serum (1:2000), anti-HSP60 serum (1:2000), affinity purified rat anti-TbACO serum (1:300), and anti-Ty1 monoclonal antibody (BB2 culture supernatant, 1:50) as indicated. Phosphoglycerate kinase B (PGK-B, detected by anti-phosphoglycerate kinase C serum) served as cytosolic marker and mitochondrial heat shock protein HSP60 served as mitochondrial marker (41). A and B, AxTat1.1 wild type procyclic trypanosomes (2.5 × 10⁶ or 5 × 10⁶ cells for HSP60 only) per lane. C and D, transgenic MiTat1.4 procyclic trypanosomes stably expressing an epitope (Ty1)-tagged TbACO. Western blots are shown in A and C, and the corresponding quantitative data obtained by densitometric scanning are displayed in B and D. The released fraction (% release) is calculated by dividing the amount of specific protein in the supernatant by the total amount of specific protein in supernatant and pellet of the respective sample.
deviating from the preesseence consensus features.

Why do trypanosomes have a cytoplasmic IRP-like aconitase? First, it is possible that the protein serves a gene regulatory function similar to mammalian IRPs. Recently, the *Bacillus subtilis* IRP-like aconitase has been shown to bind to mammalian IREs (94). The sequence motif DLVIDH-IQV implicated in RNA binding of IRP (95) is nearly conserved in TbACO (three conservative substitutions); however, no sequence-specific binding of the mammalian IRE consensus RNA sequence to recombinant purified TbACO could be detected. The putative trypanosomal target RNA sequence may deviate, and thus a regulatory role can only be addressed genetically. We have investigated expression and regulation of one possible target, the trypanosomal transferrin receptor, and found no change of its expression or regulation in cells carrying a targeted deletion of TbACO (96). Second, we have considered the possibility that TbACO serves as cytoplasmic iron store. In fact, the IRP-related aconitase of *L. pneumophila* is the major iron-containing protein in that organism (54). However, a calculation based on the iron uptake rate of trypanosomes (97) showed that TbACO should contribute less than 10% to the total iron content of proliferating slender bloodstream forms. Given the abundance of cytoplasmic TbACO, particularly in the procyclic stage, a metabolic function seems the most likely. In plant tissues, cytoplasmic aconitase is developmentally regulated with a dramatic increase during seed and pollen maturation and during germination, reflecting glyoxalate cycle activity (reviewed in Ref. 19). Evidence for a glyoxalate cycle in stationary promastigotes (probably metacyclic forms) of the trypanosomatid *Leishmania* has been reported (98, 99). Although key enzymes of the glyoxalate cycle have not been detected in procyclic forms of *T. brucei* in culture, a functional glyoxalate cycle may be operative and important at some later stage in the insect vector, where utilization of storage lipids may compensate for temporary shortage of nutrients. We are currently testing this hypothesis by tsetse fly passage of TbACO knock out strains.

Citic acid cycle activities and certain respiratory chain activities are turned on in the stumpy bloodstream stage of *T. brucei* as a preadaptation to the tsetse midgut environment, where rapid differentiation to the procyclic stage is essential for survival (5, 8). The developmental profile of TbACO expression fully accounts for the previously reported changes of total aconitase activity (5, 7) and parallels the developmental changes in energy metabolism in the trypanosomal life cycle (1, 2). More than 30-fold developmental regulation of TbACO contrasts with only a minor change of mRNA abundance, indicating a translational or post-translational mechanism. The only other citric acid cycle enzyme that has been cloned so far, malate dehydrogenase, seems to be regulated in a similar fashion (100). We anticipate that a common translational or post-translational mechanism may coordinate up-regulate citric acid cycle activities upon differentiation. Whereas numerous examples suggest a predominance of regulation at the level of differential mRNA stability in trypanosomatids (101, 102), only a handful of examples of translational or post-translational regulation have been reported (103–107).

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