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The relation between the soluble factor associated with H^+-transhydrogenase of *Rhodospirillum rubrum* and the enzyme from mitochondria and *Escherichia coli*

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Although in mitochondria, *Escherichia coli* and *Rhodobacter capsulatus* the H^+-transhydrogenases are intrinsic membrane proteins, in *Rhodospirillum rubrum* a water-soluble component (Th_s) and a membrane-bound component are together required for activity. Th_s was selectively removed from chromatophore membranes of *Rhs. rubrum* and was purified to homogeneity by precipitation with $(NH_4)_2SO_4$ and ion-exchange, affinity dye and gel exclusion chromatography. The latter indicated an M_r of approx. 74000 under non-denaturing conditions but analysis of the pure protein by SDS-PAGE revealed a single polypeptide, M_r 43000. Antibodies against this polypeptide inhibited transhydrogenase activity of chromatophores and decreased the capacity of Th_s to restore activity to depleted membranes. They reacted with a polypeptide of M_r 43000 in crude cell extract, chromatophore membranes and chromatophore washings but not with transhydrogenase polypeptides from the membranes of *E. coli*, *Rb. capsulatus* or animal mitochondria. The N-terminal amino acid sequence of the 43000 polypeptide was strongly homologous with the reported N-terminal regions of mitochondrial transhydrogenase and the α subunit of the *E. coli* protein. The break between the α and β polypeptides of *E. coli* transhydrogenase is such that both components are membrane-associated. In contrast, these results suggest that in the *Rhs. rubrum* enzyme Th_s has been formed by a break closer to the N-terminus, thus avoiding the putative trans-membrane helical segments and yielding a relatively hydrophilic subunit, which is water-soluble. There is a predicted similarity between Th_s and the reported sequence of alanine dehydrogenase from *Bacillus* but Th_s did not have any alanine dehydrogenase activity.

Introduction

H^+-Thase is an enzyme, located in the membranes of mitochondria and some bacteria, which couples the translocation of protons to the transfer of H^- equivalents between NAD(H) and NADP(H):

$$H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH$$

(for reviews, see [1,2]). The mitochondrial H^+-Thase comprises a single polypeptide, M_r 109000 [3] and that from *Escherichia coli*, two polypeptide: alpha, M_r 54000, and beta, M_r 49000 [4]. Both proteins probably function as dimers [5,6]. When the alpha and beta polypeptides of *E. coli* are arranged contiguously there is considerable homology with the mitochondrial protein. The single polypeptide of mitochondrial H^+-Thase consists of three domains, a predominantly hydrophilic N-terminal region (domain I), a central sequence (domain II) of mainly hydrophobic amino acids (possibly 14 trans-membrane helices) and a predominantly hydrophilic C-terminal region (domain III). The 'break' between the two polypeptides of the *E. coli* protein lies in domain II. Thus, the mitochondrial polypeptide and both the *E. coli* polypeptides are membrane proteins and require detergent for their solubilisation and stability [7–9].

Fisher and colleagues found that the H^+-Thase activity of everted membrane vesicles (chromatophores) from the photosynthetic bacterium, *Rhodospirillum*

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Abbreviations: H^+-Thase, H^+-translocating NAD(P)H transhydrogenase; Th_s, Th_m, soluble and membrane-bound components, respectively, of transhydrogenase from *Rhodospirillum rubrum*; C_T-particles, chromatophores depleted of the soluble transhydrogenase component; DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride; BME, β-mercaptoethanol; AcPdAD$^+$, acetylpyridine adenine dinucleotide.

Enzyme: H^+-translocating NAD(P)H transhydrogenase (EC 1.6.1.1).

rubrum. was lost upon washing by centrifugation through low ionic strength buffer [10–15]. Activity was completely restored upon addition of an $(NH_4)_2SO_4$ precipitate of the supernatant fraction. It was suggested that a 'soluble factor', Th_s, in the precipitate is a component of the transhydrogenase enzyme. The depleted chromatophores were presumed to possess a complementary intrinsic membrane component, Th_m. The H^+-Thase of chromatophores from other photosynthetic bacteria (e.g., *Rhodobacter capsulatus* and *Rb. sphaeroides*) can not be resolved in this way [16,17]. In fact, the polypeptide composition of the the enzyme from *Rb. capsulatus* resembles that from *E. coli* [17]. In principle, Th_s from *Rhs. rubrum* might correspond to one or both of the relatively hydrophilic regions of H^+-Thase from mitochondria and *E. coli* or it might be an unrelated protein that is required for activation. In this report we describe the purification, polypeptide composition and N-terminal sequence of Th_s from *Rhs. rubrum* and consider the relationship between this protein and H^+-Thase from other organisms.

Methods

Rhs. rubrum strain S1 (from Dr. L. Slooten, Vrije Universiteit, Brussel) was grown anaerobically in the light to late exponential phase in 'RCV medium' [18] supplemented with 15 $\mu g\ l^{-1}$ biotin, as described for *Rb. capsulatus* [19]. The harvested cells were washed in 10% (w/v) sucrose, 100 mM Tris-HCl (pH 8.0), resuspended in the same medium and broken in an Aminco French Press at 9000 psi. The membrane fraction sedimenting between $35000 \times 30\ g \cdot min$ and $100000 \times 180\ g \cdot min$, resuspended again in the same medium (plus 50% glycerol, v/v), was taken as 'chromatophores'. The bacteriochlorophyll concentration was estimated from the in vivo extinction coefficient $E^{880} = 140\ mM^{-1}\ cm^{-1}$ [20].

Crude Th_s was prepared from the cell extract (the supernatant remaining after the high-speed centrifugation step during the preparation of chromatophores) by collecting the fraction precipitating between 50% and 65% saturation with $(NH_4)_2SO_4$ and resuspending in 1% (w/v) sucrose, 10 mM Tris-HCl (pH 8.0) [11]. C_T-particles (chromatophore membranes depleted of soluble factor [10]) were prepared by washing chromatophores by centrifugation (four times) through 10% sucrose, 100 mM Tris-HCl (pH 8.0). Crude Th_s was also prepared from the pooled washings by collecting the fraction precipitating between 40% and 90% saturation with $(NH_4)_2SO_4$ and resuspending in 1% sucrose, 10 mM Tris-HCl, (pH 8.0).

Purified Th_s was prepared as follows: chromatophore membranes were washed once by centrifugation (at approx. 130 μM bacteriochlorophyll) in 10% sucrose, 100 mM Tris-HCl (pH 8.0), 10 μM $NADP^+$, 2 mM $MgCl_2$. The supernatants from three subsequent washes (at approx. 200 μM bacteriochlorophyll) in 10% sucrose, 100 mM Tris-HCl, pH8.0, 1 mM DTT, were pooled and brought to 40% saturation with $(NH_4)_2SO_4$, incubated at 4°C overnight and centrifuged at $31000 \times g$ for 30 min. The supernatant was brought to 90% saturation with $(NH_4)_2SO_4$, incubated overnight and centrifuged again. The pellet was resuspended in 1% sucrose, 10 mM Tris-HCl (pH 8.0), dialysed against 10 mM Tris-HCl (pH 8.0), 10 mM $(NH_4)_2SO_4$, 1 mM PMSF, 1 mM DTT, 10 mM BME and applied to a QA-Trisacryl column (5.0×1.5 cm). The column was pre-equilibrated and developed with 10 mM Tris-HCl (pH 8.0), 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 10 mM BME. Th_s activity was eluted in the void volume of the column but contaminants were retarded. Active fractions were applied to a Reactive Green 19-agarose column (3.5×1.0 cm), pre-equilibrated and developed with 10 mM MES (pH 6.5), 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 10 mM BME. Following elution of inactive protein, Th_s was displaced from the column with 0.4 M $(NH_4)_2SO_4$. In some instances the active Th_s was then subjected to chromatography on a column of AcA-44 (80×2.5 cm), pre-equilibrated and developed with 10 mM Tris-HCl (pH 8.0), 100 mM $(NH_4)_2SO_4$, 1 mM DTT.

QA-Trisacryl and AcA-44 were obtained from IBF Biotechnics and Reactive Green 19-agarose from Sigma.

H^+-Thase activity in chromatophores and in C_T-particles reconstituted with preparations of Th_s was assayed at 30°C by monitoring the reduction of thio-$NADP^+$ at 395–450 nm ($E = 10.65\ mM^{-1}\ cm^{-1}$) in a Shimadzu UV-3000 dual-wavelength spectrophotometer. Saturating photosynthetic excitation was provided with a high-power GaAlAs emitter [21]. The medium (3 ml) contained 125 mM sucrose, 2.67 mM $MgCl_2$, 44 mM Tris-HCl (pH 8.0), 33 μM thio-$NADP^+$, 133 μM NADH, either chromatophores or C_T-particles to give 6.7 μM bacteriochlorophyll and an appropriate quantity of Th_s.

Since, on its own, highly purified Th_s had no detectable transhydrogenase activity (see below), the assay of activity during purification was carried out by adding test fractions to a suspension of C_T-particles and measuring the rate of reduction of thio-$NADP^+$ by NADH in this reconstituted system during photosynthetic illumination. Generally, for the detection of Th_s protein, aliquots from different fractions were compared only on a qualitative basis. For quantitative comparison, the amount of Th_s was estimated by the incremental increase in transhydrogenase activity when the amount of C_T-particles was in large excess.

Experiments to measure the transhydrogenase activity of purified Th_s in the absence of C_T-particles were performed on a Kontron Uvicon 810 spectrophotom-

eter at 375 nm in a similar medium to that described above except that the nucleotides were replaced by 100 μM AcPdAD$^+$ and 200 μM NADPH.

SDS-PAGE (10–20% gradients) was performed [17] and protein bands were visualised by either PAGE-blue 83 or $AgNO_3$ staining. Iso-electric focussing gels were run on a Pharmacia Phast system, according to the manufacturer's instructions.

Polyclonal antibodies were raised to polypeptide eluted from SDS-PAGE, as described [17]. Western blots were also performed, as in Ref. 17, but using a 'semi-dry' electro-blotter (Millipore), which gave better transfer of high molecular weight polypeptides (M_r > 1000000). The guinea-pig and beef-heart mitochondria were a gift from Dr R. Sutton.

For amino acid micro-sequencing, purified preparations of Th_γ were subjected to SDS-PAGE and electro-blotted onto poly(vinylidene difluoride) membranes. The polypeptide was visualised with PAGE-blue 83, excised from the membrane and analysed in an Applied Biosystems 47A gas-phase sequencer.

Results

The data shown in Table I confirm the results of Fisher and colleagues [10–12]. The H^+-Thase activity of chromatophore membranes from *Rhs. rubrum* was depleted upon sedimentation through a medium of neutral pH and low ionic strength. Activity was restored by addition of either an $(NH_4)_2SO_4$ fraction of cell extract or an $(NH_4)_2SO_4$ fraction of the chromatophore washings, proposed by Fisher to contain 'Th_γ'. It was not unusual to observe rates in the reconstituted system that were in excess of those measured in the native chromatophores. Thus, it is probable that some Th_γ was lost from chromatophores during preparation. The addition of proteinase inhibitors (2 mM PMSF, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin and 0.5 μg ml^{-1} EDTA) to the bacterial cell suspension before French Pressure treatment did not lead to enhanced transhydrogenase activity in chromatophores. Nor did it affect the extent of loss of Th_γ during subsequent washing or the capacity for reconstitution. Generally, for a given quantity of bacterial cells, there was about three times more Th_γ activity in the chromatophore washings than in the cell extract. Moreover, analysis by SDS-PAGE showed that the latter contained a complex mixture of polypeptides whereas the composition of the former was relatively simple and thus provided a more auspicious starting material for the preparation of Th_γ.

In agreement with [11], the loss of transhydrogenase activity during centrifugation of the chromatophores was partly prevented by the inclusion of a low concentration of $NADP^+$ (not shown). The presence of Mg^{2+} also contributed to maintenance of activity on the membranes (not shown). The extent of loss in the presence of both $NADP^+$ and Mg^{2+} amongst different preparations was generally between 20–25% (but occasionally was as much as 60%). These observations led to a simple procedure for the purification of Th_γ (see Methods and also [16]). Note that the $(NH_4)_2SO_4$ precipitate of Th_γ, after being re-dissolved in 10 mM Tris-HCl and 1% sucrose, remained stable at 4°C for several days but activity was lost rapidly upon dialysing against the same medium to remove the $(NH_4)_2SO_4$. This loss of activity was evidently a combined effect of oxidation of -SH groups in the protein and activation of proteinases at low ionic strength, since it was prevented by a combination of DTT, beta mercaptoethanol and PMSF.

Th_γ was eluted from an AcA-44 gel exclusion chromatography column with an apparent M_r = 74000. It was previously shown that Th_γ activity from a crude extract was eluted from a gel filtration column in fractions corresponding to an M_r of approx. 70000 [22]. A summary of yields and activities during purification is given in Table II.

Analysis of purified Th_γ by iso-electric focussing under non-denaturing conditions gave a sharp single band, indicating an iso-electric point at pH 6.2 (not shown). There was no detectable absorbance of the protein (at 15 μg ml^{-1}) in the visible or near ultraviolet. It contains no flavin. Fig. 1 shows the polypeptide composition of purified Th_γ and of active fractions collected during the preparation, as revealed by SDS-PAGE. The protein appears to comprise a single polypeptide of apparent M_r = 43000. Evidently it constitutes only a minor component in the chromatophore washings.

In the absence of C_T-particles, purified Th_γ had no observable transhydrogenase activity (in the direction, NADPH → $AcPdAD^+$) within the limits of detection,

TABLE I

Resolution and reconstitution of light-driven transhydrogenase activity in Rhs. rubrum chromatophores

	Rate of light-driven H^+-Thase (μmol thio-$NADP^+$ reduced · min^{-1} · μmol bacteriochlorophyll^{-1})
Chromatophores	1.29
Washed chromatophores [a]	0.22
Washed chromatophores + $(NH_4)_2SO_4$ precipitate of cell extract [b]	1.64
Washed chromatophores + $(NH_4)_2SO_4$ precipitate of washings [c]	1.33

[a] Washed with 10% sucrose, 100 mM Tris-HCl, pH 8.0;
[b] 0.5 mg protein;
[c] 0.025 mg protein.

TABLE II

Purification of Th_s from Rhs. rubrum:

Light-driven transhydrogenase was assayed with thio-NADP$^+$, as described in Methods. One unit is defined as the amount of Th_s required to increase the rate of the light-driven transhydrogenase activity in C_T-particles, when the latter are in large excess, by 1 μmol thio-NADPH·μmol bacteriochlorophyll^{-1} min^{-1}.

Stage of purification	Total activity (units)	Total protein (mg)	Specific activity (units·mg protein^{-1})
Pooled supernatants from chromatophore washing	136	25.6	5.3
Dialysed $(NH_4)_2SO_4$ fraction	107	7.8	13.8
Pooled QA fractions	50	0.35	142
Pooled RG-19 fractions	44	0.06	705

the activity must be less than 0.01 μmol (mg protein)$^{-1}$ min^{-1} (compare with 8.4 μmol AcPdAD$^+$ reduction (mg Th_s)$^{-1}$ min^{-1} in the presence of C_T-particles). A titration curve for the recovery of transhydrogenase activity of C_T-particles with purified Th_s is shown in Fig. 2.

Antibodies were raised to the 43 kDa polypeptide, excised and electro-eluted from SDS-PAGE of a Th_s preparation. Immuno-blots of crude cell extract and of chromatophore washings with this antibody are shown in Fig. 3. Both revealed a cross-reacting polypeptide,

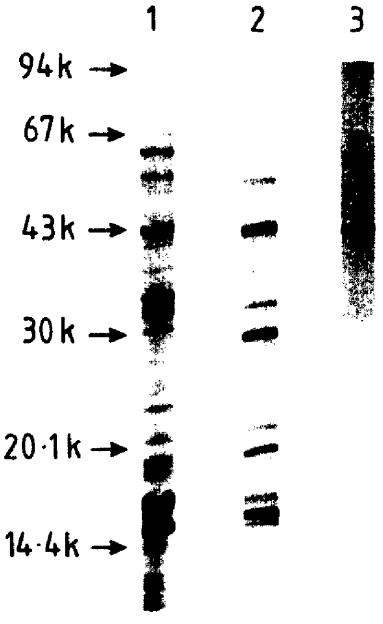

Fig. 1. Polypeptide composition of Th_s prepared from *Rhs. rubrum*. The samples were run on a 10–20% gradient polyacrylamide gel and stained with $AgNO_3$. Lanes: (1), the Th_s-active $(NH_4)_2SO_4$ precipitate from the chromatophore wash in the absence of NADP$^+$ and Mg^{2+}; (2), the pooled Th_s-active fractions after chromatography on QA-Trisacryl; (3), the pooled Th_s-active fractions after chromatography on Reactive Green 19-agarose.

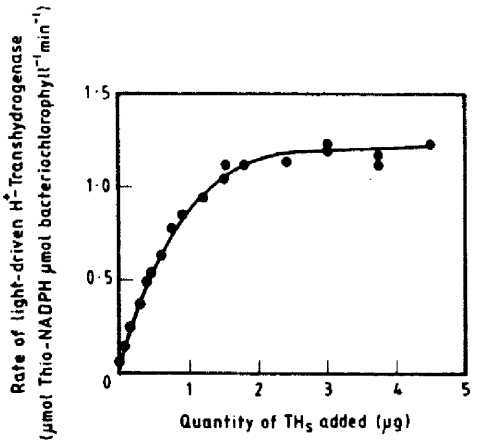

Fig. 2. Reconstitution of C_T-particles of *Rhs. rubrum* with purified Th_s. See Methods. The Th_s was prepared by the procedure described in the text, up to, and including, chromatography on Reactive Green 19-agarose.

M_r = 43000. In addition, there was a smaller cross-reacting polypeptide, possibly a proteolytic fragment in the crude cell extract. Consistent with the loss of transhydrogenase activity upon washing chromatophore membranes, reaction of the antibody was considerably diminished in C_T-particles, compared with chromatophores (Fig. 4). The antibody inhibited transhydrogenase activity in chromatophores and prevented Th_s from reconstituting activity to C_T-particles (Table III). The stimulation of transhydrogenase activity with

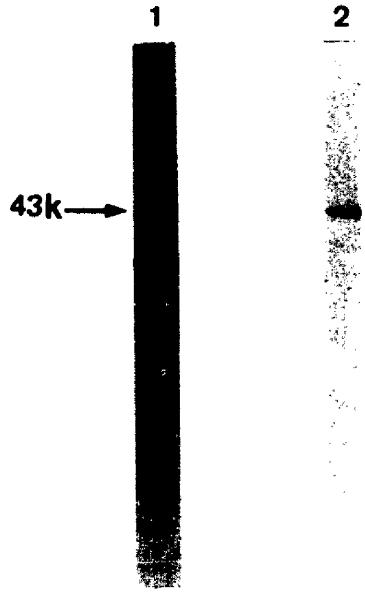

Fig. 3. Western blots of the soluble proteins of *Rhs. rubrum* with antibody against the 43 kDa polypeptide. Crude bacterial cell extract, 20 μg protein (lane 1) and chromatophore washings, 70 μg protein (lane 2), were subjected to SDS-PAGE, electro-blotted and reacted with antibody, as described in the Methods.

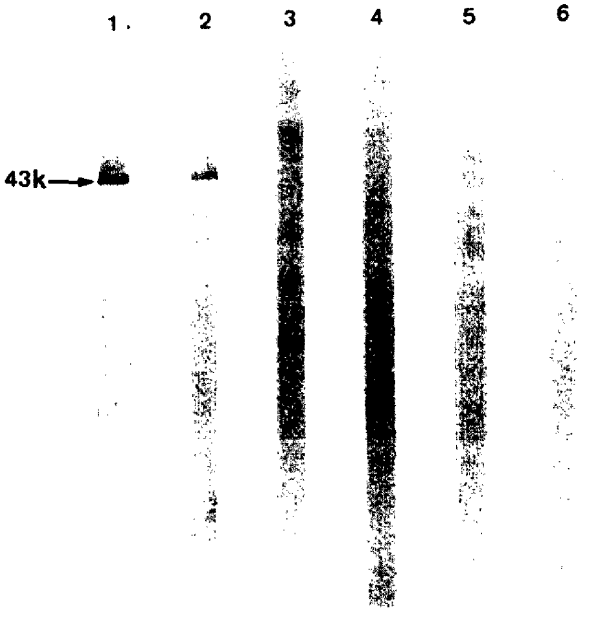

Fig. 4. Western blots of the membrane proteins of *Rhs. rubrum*, *Rb. capsulatus*, *E. coli* and mitochondria with antibody against the 43 kDa polypeptide See Methods. Lanes: (1) and (2), chromatophores and C_T-particles, respectively, from the same preparation; (3), membranes from *E. coli*, strain JM83; (4), membranes from *Rb. capsulatus*, strain SB1003; (5), guinea-pig mitochondria; (6), beef-heart mitochondria. 40 μg protein were loaded per lane.

the immunoglobulin fraction of the pre-immune serum was observed routinely but was not investigated further. The observations described above indicate that the antibody is specific in *Rhs. rubrum* for Th_γ and confirm the view that Th_γ is derived from H^+-Thase. However, under the conditions employed this antibody failed to cross-react with polypeptides known to be constituents of H^+-Thase in membranes from guinea pig or beef heart mitochondria, *E. coli* strain JM83 or *Rb. capsulatus*, strain 37b4 (Fig. 4).

The sequence of amino acids at the N-terminus of Th_γ is shown in Fig. 5. There is a strong similarity between this segment and the N-terminal region of the alpha polypeptide of H^+-Thase from *E. coli* and a region close to the N-terminus of the mitochondrial

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                  ....(14)
BOVINE HEART      LTVGVPKEIFQNEKRVALSPAGVQALVKQGFNVVVES

                    +*+*+*   ** ***+** *  *** ** *+**
                  (1)
E.coli (alpha)    MRIGIPRERLTNETRVAATPKTVEQLLKLGFTVALES

                  *+*+**+**   * ***+** *  ** *** * **
                  (1)
Rhs.rubrum (Th_S) MKIAIPKERRPGEDRVAISPEVVKKLVGLAFEVIVEQ
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Fig. 5. Amino acid sequence of the N-terminus of Th_γ. The bovine-heart and *E. coli* sequences are taken from Ref. 3 and 4. *, indicates amino acid identity and +, conservative substitutions between adjacent sequences.

TABLE III

Inhibitory effects of antibodies against the 43-kDa peptide
(i) Inhibition of transhydrogenase activity in chromatophores

Chromatophores (4.1 μM bacteriochlorophyll) were assayed for AcPdAD$^+$ reduction by NADPH, as described in Methods. Antibody or the immunoglobulin fraction of pre-immune serum was added where shown (100 μl to an assay volume of 1 ml) and incubated for 5 min at 30°C before assaying.

	Transhydrogenase activity (μmol APADH·μmol bacteriochlorophyll^{-1}·min^{-1})	
	experiment A	experiment B
Chromatophores	0.82	0.84
Chromatophores + antibody	0.47	0.49
Chromatophores + immunoglobulins	0.96	0.94

(ii) Inhibition of the capacity of Th_γ to reconstitute transhydrogenase activity to C_T-particles

Purified Th_γ (0.15 μg protein) in 860 μl assay buffer (see Methods) was incubated with 100 μl antibody or the immunoglobulin fraction of pre-immune serum or buffer for 5 min at 30°C. C_T-particles were added to 10 μM bacteriochlorophyll followed by 200 μM NADPH. 2 min later the reaction was initiated by addition of 200 μM AcPdAD$^+$.

	Transhydrogenase activity (μmol APADH·μmol bacteriochlorophyll^{-1}·min^{-1})	
	experiment A	experiment B
Th_γ	0.61	0.61
Th_γ + antibody	0.29	0.33
Th_γ + immunoglobulins	0.74	0.74

protein. 49% of residues 1–37 of Th_γ are identical with those in the equivalent region in the *E. coli* protein and 40.5% are identical with amino acids 14–40 in mitochondrial H^+-Thase. There is 49% identity be-

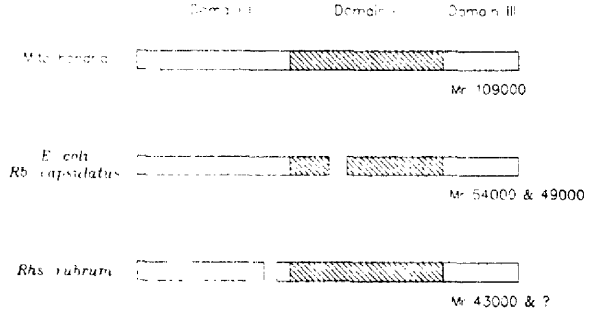

Fig. 6. Relationship between the structures of transhydrogenases from *Rhs. rubrum*, *E. coli* and bovine heart. The unshaded and shaded regions represent relatively hydrophilic and relatively hydrophobic amino acid regions, respectively. The information for mitochondrial, *E. coli* and *Rb. capsulatus* transhydrogenases was taken from Refs. 3, 4 and 17. The representation of the membrane-bound component of the *Rhs. rubrum* protein is a simple prediction on the basis of the known structure of the other transhydrogenases.

tween the *E. coli* protein and the mitochondrial protein in this region.

Discussion

Although antibodies against Th_s from *Rhs. rubrum* do not cross-react with transhydrogenase polypeptides in mitochondrial or *E. coli* membranes, the N-terminal sequence of Th_s shows clear homology with the single polypeptide of the bovine heart protein and the alpha-subunit of H^+-Thase from *E. coli*. The original observations of Fisher and colleagues [10–15] can now be understood in terms of the domain structure of the molecule. Thus, on the basis of the apparent M_r of 43000, Th_s comprises the equivalent of most of the relatively hydrophilic N-terminal region, domain I, of H^+-Thase from bovine heart and *E. coli* (Fig. 6). Th_m is presumably composed of domains II and III. Since Th_s is a readily dissociable, water soluble protein, the observations in *Rhs. rubrum*, in turn, confirm the supposition [3,4] that domain I extends from the membrane as a peripheral structure on the matrix side in mitochondria and the cytoplasmic side in *E. coli*.

There is evidence that domain I of H^+-Thase from mitochondria and *E. coli* has an NAD(H) binding site [3,4,23,24]. The observation that NAD^+ partly protected Th_s against inhibition by butanedione [14] therefore supports the view that Th_s is homologous with domain I. There is also evidence that the NADP(H) site of mitochondrial H^+-Thase is located in the predominantly hydrophilic C-terminal region, domain III [24]. This does not fit so neatly with the observed protective effects of $NADP^+$ against inhibition of Th_s by butanedione [14] and *N*-ethylmaleimide [15]. It may be that domain I of H^+-Thase from mitochondria (and *E. coli*) possesses an, as yet undetected, binding site for $NADP^+$; alternatively, the rather high concentrations of $NADP^+$ required in the Th_s protection experiments [14,15] may indicate that the effects were not associated with the catalytic binding site for NADP(H) on the enzyme.

The H^+-Thases of mitochondria and of *E. coli* are reported to function as dimeric structures [5,6]. Comparison of the apparent M_r values on SDS-PAGE (43000) and under non-denaturing conditions on gel filtration (74000), this work and Ref. 22, suggests that Th_s may exist as a dimer and this may reflect the situation in the native enzyme. A 43000 fragment isolated from the N-terminus of mitochondrial H^+-Thase by treatment with trypsin also appeared to be dimeric [25]. Like Th_s, the N-terminal fragment of the mitochondrial protein retained an NAD^+ binding site [25].

The part of the amino acid sequence of mitochondrial [3] and *E. coli* [4] H^+-Thase which is expected to correspond with Th_s is very similar to the sequence of alanine dehydrogenase, a 'soluble' protein from *Bacillus stearothermophilus* and *B. sphaericus* [26]. Thus, an alignment (see [29]) of the 409 amino acids from the N-terminus of H^+-Thase from bovine mitochondria with the 372 residues of alanine dehydrogenase of *B. sphaericus* has a sequence identity of 30.7% (not shown). The similarity is not confined to the postulated nucleotide binding regions. Since Th_s can exist as a stable entity, independently of the membrane component, it might indicate interesting relationships in the 3-dimensional structures but it does not necessarily have any mechanistic implications. Purified preparations of Th_s had no detectable alanine dehydrogenase activity when assayed in the direction of amination, with either NADH or NADPH, or in the direction of deamination with NAD^+ using the assay conditions described in Refs. 27 and 28.

It is possible that, in vivo, the H^+-Thase of *Rhs. rubrum* exists as a membrane-bound protein like that in mitochondria and *E. coli* and that during disruption of the bacteria it is cleaved by a protease that is insensitive to the inhibitors PMSF, leupeptin, pepstatin and EDTA. Cleavage would not, itself, cause enzyme inactivation but would merely permit reversible dissociation of Th_s from Th_m. Since the components can re-assemble in vitro to give an active enzyme, despite the 'loss' of this peptide bond, a simpler view is that Th_s is not covalently linked to Th_m, even in the intact cell. Whichever alternative is correct, the fact that a functionally-intact NAD(H)-binding domain can be separated from the protein in a soluble form could be of great value in determining the mechanism of action of H^+-Thase.

Acknowledgements

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