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Estimation of the H^+/H^- ratio of the reaction catalysed by the nicotinamide nucleotide transhydrogenase in chromatophores from over-expressing strains of *Rhodospirillum rubrum* and in liposomes inlaid with the purified bovine enzyme

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Abstract

Two strains of *Rhodospirillum rubrum* were constructed in which, by a gene dosage effect, the transhydrogenase activity of isolated chromatophores was increased 7–10-fold and 15–20-fold, respectively. The H^+/H^- ratio (the ratio of protons translocated per hydride ion equivalent transferred from NADPH to an NAD^+ analogue, acetyl pyridine adenine dinucleotide), determined by a spectroscopic technique, was approximately 1.0 for chromatophores from the over-expressing strains, but was only approximately 0.6 for wild-type chromatophores. Highly-coupled proteoliposomes were prepared containing purified transhydrogenase from beef-heart mitochondria. Using the same technique, the H^+/H^- ratio was close to 1.0 for these proteoliposomes. It is suggested that the mechanistic H^+/H^- ratio is indeed unity, but that a low ratio is obtained in wild-type chromatophores because of inhomogeneity in the vesicle population.

Keywords: Proton/hydride ratio; Nicotinamide nucleotide transhydrogenase; Hydride ion transfer; Chromatophore; Liposome; Overexpression

1. Introduction

The nicotinamide nucleotide transhydrogenase, found in the inner membrane of animal mitochondria and the cytoplasmic membrane of many bacteria (for recent reviews, see [1–3]), couples the transfer of hydride ion equivalents between NAD(H) and NADP(H) to the translocation of protons:

$$NADH + NADP^+ + xH^+_{out} \Leftrightarrow NAD^+ + NADPH + xH^+_{in}$$

where x is the H^+/H^- ratio, the stoichiometry of H^+ translocated across the membrane for each H^- equivalent transferred between NAD(H) and NADP(H). In its forward direction (left to right in the equation), the operation of transhydrogenase leads to the consumption of the transmembrane proton electrochemical gradient (Δp) generated by electron-transport reactions.

The physiological role of transhydrogenase is not fully established: among others, NADPH production and Δp generation were suggested as possible functions (reviewed in [4]). It is possible that in mitochondria the enzyme has primarily a regulatory role, operating in a substrate cycle with isocitrate dehydrogenases, as described in [4] and recently supported by experiments described in [5].

The determination of the H^+/H^- ratio is essential for a proper understanding of the mechanism of the reaction catalysed by the protein, and it is important for the evaluation of its metabolic role. For example, the mass action ratio ([NADPH][NAD^+]/[$NADP^+$][NADH]) and even the direction of the reaction catalysed by transhydrogenase under physiological conditions will depend on this stoichiometry [4].

Measurements of the H^+/H^- ratio, carried out on different systems (liposomes and everted membrane vesicles), and with different sources of enzyme (from beef-heart mitochondria and from two species of photosynthetic bacteria), have generally given values in the range of 0.35–1.0

Abbreviations: $AcPdAD^+$, acetyl pyridine adenine dinucleotide; thio-$NADP^+$, thionicotinamide adenine dinucleotide phosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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[6–14]. In view of this broad spread of results, it has been difficult to discriminate between two possible mechanistic ratios, 0.5 and 1.0. A detailed review of these experiments was given in a previous publication [14], but some points should be mentioned here.

In the earlier work by Fisher and co-workers [8–11], purified transhydrogenase from beef heart mitochondria was reconstituted into liposomes and the H^+/H^- ratio was estimated from parallel experiments: the rate of proton uptake from the medium into proteoliposomes during reverse transhydrogenase reaction was measured with a glass electrode, and the rate of H^- transfer between nucleotides, by spectroscopy. The major limitation of this procedure was that the slow response of the glass electrode prevented the measurement of initial rates of proton uptake (rates were recorded about 10 s after the start of the reaction). Estimation of the H^+/H^- ratio could only be made by extrapolation to zero time; values between 0.35 and 0.84 were obtained [6–9], and a mechanistic ratio of 1.0 was proposed. The reliability of the extrapolation depends critically on the nature of the non-specific leak of protons through the membrane of the liposomes.

Rydstrom and colleagues co-reconstituted mitochondrial transhydrogenase and H^+-ATPase into liposomes and, from the rate of the ATP-driven transhydrogenase reaction, estimated that the H^+/H^- ratio was about 1 [10]. However, because the amount of non-specific proton leak was not determined in these experiments, it was acknowledged [10] that the calculated ratio was an upper limit. Furthermore, the calculation was based on the assumption that the H^+/ATP ratio for ATPase is 3.0; some recent estimates put the value of this ratio at 4.0 [15].

Chromatophores (everted membrane vesicles) from photosynthetic bacteria (*Rhodobacter capsulatus* and *Rhodospirillum rubrum*) were used by our group to estimate the H^+/H^- ratio. In a series of experiments, in which either Δp or the membrane ionic current were measured using electrochromic absorbance changes of endogenous carotenoids, values of 0.4 ± 0.5 [11], 0.72 [12] and 0.55 [13] for the ratio were obtained. Recently, we estimated the H^+/H^- ratio in chromatophores from *Rhs. rubrum* by a similar procedure to that employed by Fisher et al. [6–9], but where the time resolution for the measurement of proton uptake was improved by using rapid mixing devices and a pH indicator, Cresol red, instead of a glass electrode [14]. Furthermore, the rate of proton translocation was corrected for leak from measurements of proton efflux following short periods of photosynthetic illumination. An H^+/H^- ratio of 0.60 ± 0.06 was obtained.

The implications of this result, that the enzyme might 'slip' or that the mechanistic ratio might be 0.5, were discussed [14], but the discrepancy with earlier results using the mitochondrial enzyme was puzzling. In particular, although the data of Fisher et al. were somewhat scattered [6–9], for some proteoliposome preparations the estimated value of the H^+/H^- ratio seemed to be significantly greater than 0.5, and their method is expected to lead to an *underestimate* of the true ratio (see above). In the present report we describe the results of experiments that were set up in an attempt to resolve the discrepancy. We have developed strains of *Rhs. rubrum* in which the gene for transhydrogenase is over-expressed. Chromatophores isolated from these strains have a substantially higher rate of the transhydrogenation reaction than those prepared from wild-type bacteria and the accompanying proton uptake rate can be measured with greater accuracy. We have also used proteoliposomes inlaid with mitochondrial transhydrogenase. These have a slightly lower rate of proton leak (on the basis of the degree of stimulation of the transhydrogenase reaction with uncoupler) than those used by Fisher et al. Throughout, we have used the procedure that was introduced by Fisher's group [6–9] for the measurement of H^+/H^- ratio, but with the important modification [14] that proton uptake was measured spectrophotometrically, with better time resolution.

2. Materials and methods

2.1. Materials

Egg-yolk phosphatidylcholine and lysophosphatidylcholine were obtained from Lipid Products (UK), endonuclease restriction enzyme, ligase and alkaline phosphatase were from Northumbria Biologicals. All other chemicals were from Sigma unless otherwise stated.

The bacterial strains and plasmids used in this work are shown in Table 1.

2.2. Growth conditions

E. coli strains were grown aerobically with vigorous shaking in LB medium at 37°C [16]. *Rhs. rubrum* strains were grown anaerobically in the light at 30°C, on 'RCV medium *plus* biotin' in completely sealed bottles, as described [17]. When used for the preparation of chromatophores, *Rhs. rubrum* strains were grown in RCV *plus* biotin medium supplemented with 0.1% yeast extract. Where appropriate, media were supplemented with either 10 μg/ml of tetracycline (strains harbouring pNIC2 or pRK415) or 25 μg/ml of kanamycin (strains harbouring pNIC3 or pKT230). Solid media were prepared using the liquid media described above but supplemented with 1.5% Agar (Difco).

2.3. Construction of plasmids

Isolation of plasmid DNA, bacterial transformation, restriction endonuclease digestion, DNA dephosphorylation and ligation, agarose gel electrophoresis, Southern blotting and other standard techniques were performed as described [16]. pNIC2 was obtained by inserting the 6.1 kb *Bam*HI

fragment of pNIC1 [18] bearing the genes coding for *Rhs. rubrum* transhydrogenase into the *Bam*HI site of pRK415 (Fig. 1). pNIC3 was obtained by inserting the 6.1 kb *Bam*HI fragment of pNIC1 into the *Bam*HI site of pKT230 (Fig. 1). Following the construction of these plasmids in *E. coli* strain MC1061, they were used for the transformation of strain S17-1.

2.4. Mating procedure

Plasmids were transferred from *E. coli* strain S17-1 into *Rhs. rubrum* by conjugation using the mating procedure described [19]. A suspension (0.1 ml) of *E. coli* in mid-exponential phase ($A_{650} \sim 0.3$) was mixed in a microcentrifuge tube with 0.1 ml of a late exponential phase phototrophic culture of *Rhs. rubrum* ($A_{650} \sim 1.5$), and centrifuged. The pelleted cells were resuspended in 10 μl of RCV *plus* biotin medium, spread on a sterile nitrocellulose filter (25 mm, 0.45 μm pore size, Whatman) which was placed on YPN solid medium (0.3% yeast extract, 0.2% bactopeptone, 0.5% NaCl and 1.5% Agar, pH 6.8) and incubated for 24 h aerobically in the dark at 30°C. The bacteria were removed from the filter by washing with 1 ml of RCV *plus* biotin medium and diluted (10^2- to 10^4-fold). Cells of *Rhs. rubrum* harbouring plasmids were selected by spreading the diluted suspension on RCV *plus* biotin solid medium in the presence of the appropriate antibiotic and subsequently growing on under photoheterotrophic conditions at 30°C. Plasmid DNA was isolated from individual clones, treated with restriction enzymes and examined by agarose gel electrophoresis. The presence of the plasmid bearing the transhydrogenase genes was confirmed by Southern blot analysis using a ^{32}P-labelled probe prepared from the *Bam*HI-*Eco*RI fragment of *pntAA* from pNIC1.

2.5. Long-term storage of strains

The wild-type strain of *Rhs. rubrum* was stored in 'stabs' of solid RCV *plus* biotin medium (see above). To minimise plasmid loss (e.g., see [20]) it was desirable to maintain the over-expessing strains at −80°C but, in our hands, *Rhs. rubrum* did not always remain viable when conventional low-temperature storage procedures were employed. However, the following protocol (based on [21], and devised by N.P.J. Cotton, personal communication) was found to be satisfactory. Mid- to late-exponential phase phototrophic cultures (30 ml) were centrifuged (4000×15 $g \cdot$ min) at room temperature. The pelleted cells were resuspended in 2 ml RCV *plus* biotin medium containing 10% glycerol, kept on ice for 30 min, dispensed into aliquots, and then placed at −80°C. To revive, the suspensions were allowed to thaw slowly before being added to growth medium at a dilution of at least 200-fold.

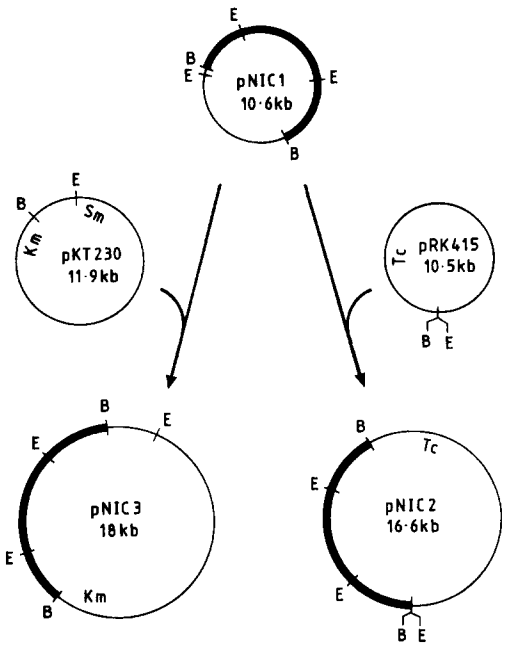

Fig. 1. The plasmid constructs used to generate the transhydrogenase over-expressing strains of *Rhs. rubrum*. Km, kanamycin; Sm, streptomycin; Tc, tetracycline; B, *Bam*HI; E, *Eco*RI.

Table 1
Bacterial strains and plasmids used in this work

Species	Strain	Relevant characteristic	Ref.
E. coli	MC1061	*hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU⁻ galK⁻ rpsL thi*	[35]
E. coli	S17-1	*recA, thi, pro, hsdR⁻ M⁺*, < RP4:2-Tc: Mu-:Km:Tn7, Tp^R, Sm^R, integrated into the chromosome >	[36]
Rhs. rubrum	S1	wild-type laboratory strain from Dr. L. Slooten	

Plasmid	Markers	Size (kb)	Ref.
pNIC1	ampicillin	10.5	[18]
pNIC2	tetracycline	18	this work
pNIC3	kanamycin	16.5	this work
pKT230	kanamycin, streptomycin	11.9	[34]
pRK415	tetracycline	10.5	[27]

2.6. Chromatophores preparation

Chromatophores were prepared from photosynthetically grown cells of *Rhs. rubrum* as described [17], and washed by centrifugation in a medium containing 50 mM KCl, 1 mM $MgCl_2$, 40 μM $NADP^+$ to decrease the buffer concentration to around 2 mM Tris-HCl. Vesicle concentrations were based on the measurement of bacteriochlorophyll concentrations using the in vivo absorption coefficient at 880 nm of 140 mM^{-1} cm^{-1} [22].

2.7. Reconstitution of mitochondrial transhydrogenase

Transhydrogenase from beef heart mitochondria was purified to homogeneity using ion-exchange chromatography as described elsewhere [23]. Enzyme (specific activity 30 μmol min^{-1} (mg protein)$^{-1}$, measured as described [23]) was stored in small aliquots of 5 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.025% Triton X-100 (pH 7.0) at 0.025 mg protein/ml at −80°C. A solution of phosphatidylcholine (25 mg) in chloroform was dried under nitrogen, washed in diethyl ether, resuspended in 2.25 ml of 100 mM potassium phosphate, 100 mM KCl (pH 7.0) and sonicated for 10 min in an ice-cold water-bath. All further steps were performed at 4°C. Thawed transhydrogenase solution (1.5 ml) and 0.15 ml of 10% Triton X-100 were added to the sonicated lipid suspension and the mixture was incubated for 10 min under constant stirring. At this stage, the lipid/protein ratio (mass/mass) was about 400, and the lipid/detergent ratio, about 1. Bio-Beads SM-2 (Bio-Rad), extensively washed in methanol and water, were used to remove detergent. Three additions of Bio-Beads, each of 150 mg (wet weight), were made at 2 h intervals to provide for slow removal of detergent, as suggested in [24]. The suspension of proteoliposomes was further dialysed for 4 h against 1.5 l of 50 mM KCl (pH 7.0), to remove buffer from the medium external to the liposome membrane. The preparation was stable for at least 10 h when kept on ice.

2.8. Measurement of transhydrogenase activities

'Forward' and 'reverse' transhydrogenation activities were measured as described [13,17,23].

2.9. H^+/H^- ratio measurements

Experiments to measure the rate of transhydrogenation and the accompanying proton uptake were performed consecutively, under identical conditions, in a clear plastic 1 × 1 cm cuvette (4 ml) with a Shimadzu UV-3000 dual-wavelength spectrophotometer. Rapid mixing was effected by injection into the cuvette from a Hamilton syringe while stirring mechanically with a propeller driven by a vertically mounted 12 V DC motor. Mixing was more than 90% complete in approximately 400 ms [25].

Transhydrogenation activity was measured by following the reduction of $AcPdAD^+$, an NAD^+ analogue, with NADPH at the wavelength pair 375–455 nm (absorption coefficient for the reaction: 6.1 mM^{-1} cm^{-1} [13]). We have not subtracted the 'valinomycin-independent' rate of transhydrogenation, as described [8,9], since we think that this is unjustified [14]. Anyway the correction is rather small (< 10%). Proton uptake from the medium into the liposomes or chromatophores was measured by following the absorbance changes of 67 μM Phenol red or Cresol red at the wavelength pair 560–640 nm (experiments at pH 7.0 and 7.3, respectively) or of 37 μM Chlorophenol red at 570–660 nm (experiments at pH 6.3). These pH indicators have been shown previously not to bind significantly to biological membranes and to respond only to the external medium pH [26]. Calibration of the quantity of proton uptake was made by duplicate or triplicate additions of standard HCl solution, prepared from standard 1 M HCl stock (M&B Volucon). Dilution artefacts were corrected by addition of equivalent volumes of water. It was established that the absorbance change of the pH indicator was proportional to the quantity of H^+ added across the range of the proton uptake measurement.

The proton leak of the chromatophore membranes was measured after short flash excitation using a single-beam spectrophotometer constructed in-house as described in [14].

3. Results

3.1. Characteristics of the overexpressed Rhs. rubrum strains RTB2 and RTB3

The preliminary objective of this work was to develop strains of *Rhs. rubrum* with higher levels of transhydrogenase activity than in the wild-type organism. The strategy was to sub-clone the *Rhs. rubrum* transhydrogenase genes from pNIC1 [18] into the promiscuous plasmids pRK415 (a derivative of RK2 from the P-1α incompatibility group) and pKT230 (a derivative of RSF1010 from the IncQ/P4 incompatibility group), and transfer the resulting constructs back into *Rhs. rubrum* by conjugation (Fig. 1). The copy number for RK2 derivatives in *Rhs. rubrum* is in the region of 11–14 [20] and is reportedly high also for RSF1010 derivatives in Gram-negative bacteria, for example in *Pseudomonas syringae* [27]; we thus may expect to obtain higher levels of transhydrogenase by a gene-dosage effect. In *E. coli*, up to 70-fold over-expression of transhydrogenase was achieved from a pUC-based plasmid [28]. The restriction maps and the Southern analysis of plasmid preparations derived from the transconjugant strains RTB2 and RTB3 indicated that they had aquired the constructs pNIC2 and pNIC3, respectively (data, not shown).

In chromatophores from wild-type cells of *Rhs. rubrum*, the rate of 'reverse' transhydrogenation (measured as the

reduction of AcPdAD$^+$ by NADPH) in the dark, was typically about 2 μmol (μmol bacteriochlorophyll)$^{-1}$ min^{-1}, and it was stimulated 1.5-fold, or less, by the uncoupler, FCCP. In membranes prepared from the transconjugant strains, RTB2 and RTB3, reverse transhydrogenation was approximately 7–10-fold and 15–20-fold greater, respectively, than in wild-type, and FCCP stimulated the rate of reaction by about 2-fold, indicating that the enzyme is operating electrogenically. The light-driven 'forward' reaction (e.g., reduction of thio-NADP$^+$ by NADH) in wild-type chromatophores was typically about 0.6 μmol (μmol bacteriochlorophyll)$^{-1}$ min^{-1} and it was enhanced in RTB2 and RTB3 chromatophores in roughly equivalent proportion to the 'reverse' reaction. This shows that at least some of the over-expressed transhydrogenase is located in the membrane in such a way that it can be driven by the Δp generated by photosynthetic electron transport. Note that the protein/bacteriochlorophyll ratio was similar in chromatophores of the two transconjugant strains to that in wild-type, and that, in contrast to over-expressing strains of *E. coli*, [28] negatively-stained electron micrographs of the bacteria did not reveal any structural abnormalities.

In chromatophores prepared from wild-type *Rhs. rubrum*, the NAD(H)-binding domain (domain I) of transhydrogenase can be removed as a water-soluble polypeptide, called Th$_s$. Activity can be restored to the depleted membrane components by adding back purified Th$_s$ [17,25]. When RTB2 and RTB3 chromatophores were washed in 2 M NaCl to remove Th$_s$ [29], the rate of 'reverse' transhydrogenation was decreased by 80%, but activity was completely restored by adding back-purifed, wild-type Th$_s$ prepared from the recombinant gene expressed in *Escherichia coli* [30]. This clearly indicates that the elevated transhydrogenation activity in the transconjugant strains is derived from the same membrane protein as that found in wild-type cells.

3.2. Measurement of the H^+/H^- ratio, (a) in chromatophores from the over-expressing strains of Rhs. rubrum

Experiments were carried out as described [14] but using chromatophores prepared from the transconjugant strains RTB2 and RTB3 of *Rhs. rubrum*. The reactions, in weakly buffered medium, were carried out in presence of 1 μg/ml rotenone to inhibit the NADH dehydrogenase, 0.2 μM venturicidin to decrease the proton leak of the chromatophores by blocking the proton channel of the ATP synthase, and valinomycin to dissipate the electrical component ($\Delta\psi$) of Δp. The chromatophores were incubated for 5 min in the presence of valinomycin to ensure K$^+$ equilibration before starting the reaction (by addition of AcPdAD$^+$ in the presence of NADPH). In consecutive experiments on different samples but performed under identical conditions, proton uptake was measured from the absorbance change of added pH indicator, and transhydrogenation was measured from the absorbance change due to the formation of AcPdADH. Results using RTB2 chromatophores are shown in Fig. 2. Initially, in the Cresol red traces, there was a small artefactual absorbance decrease, due to dilution and medium acidification by the AcPdAD$^+$, and this was followed by an absorbance increase as the chromatophores accumulated H$^+$ from the external medium (trace B). The proton uptake rate was initially very rapid, but declined over a period of a few seconds. By comparison with experiments performed in a stopped-flow mixing device, it was shown for wild-type chromatophores [14] that initial rates measured in this type of experiment are not an underestimate. As described for wild-type chromatophores [14], no H$^+$ uptake was observed in the absence of AcPdAD$^+$ or NADPH, or in presence of nigericin (trace D), or an impermeant buffer such as Mops and Mes (trace C), showing that the pH indicator responded only to the external pH changes. The rate of formation of AcPdADH also declined during the course of the reaction

Table 2
The H^+/H^- ratio of chromatophores from *Rhs. rubrum*, and liposomes inlaid with transhydrogenase from beef-heart mitochondria

Preparation and pH	Rate of transhydrogenation	Rate of proton uptake	Leak	H^+/H^- ratio
Chromatophores	(μmol (μmol bacteriochlorophyll)$^{-1}$ min^{-1})			
RTB2 prep. I, pH 7.3	13.7 ± 0.4 (4)	12.1 ± 0.6 (7)	1.1	0.96 ± 0.07
RTB2 prep. II, pH 7.3	17.7 ± 1.2 (3)	14.2 ± 1.3 (7)	1.0	0.86 ± 0.13
RTB2 prep. II, pH 6.3	10.5 ± 0.8 (2)	9.4 ± 0.5 (3)	n.d	0.89 ± 0.11
RTB3 prep. I, pH 6.3	40.5 ± 1.5 (3)	40.7 ± 2.2 (3)	1.2	1.03 ± 0.09
Proteoliposomes	(μmol (mg protein)$^{-1}$ min^{-1})			
pH 7.0	5.7 ± 0.3 (3)	5.75 ± 0.22 (3)	n.d.	1.0 ±0.1

The values show standard deviations with numbers of experiments performed shown in parentheses.
n.d. = not determined.

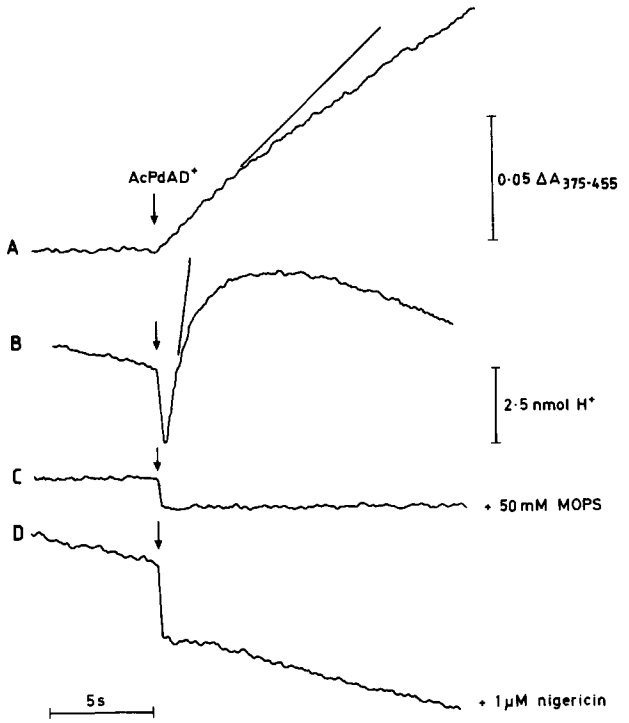

Fig. 2. The kinetics of transhydrogenation and of proton uptake by *Rhs. rubrum*, strain RTB2. Chromatophores of *Rhs. rubrum* strain RTB2 were suspended in 50 mM potassium chloride, 150 μM NADPH, 67 μM Cresol red, 0.2 μg ml^{-1} venturicidin, 1.0 μg ml^{-1} rotenone and 1.0 μg ml^{-1} valinomycin to a bacteriochlorophyll concentration of 6.5 μM and final pH 7.3. $AcPdAD^+$ was added to a concentration of 100 μM where indicated. Trace A shows the reduction of $AcPdAD^+$ measured at 375–455 nm. Traces B–D show absorbance changes at 560–640 nm. Traces C and D were taken from experiments in which the medium was supplemented with either MOPS or nigericin, as shown. The vertical bar next to trace B shows the acid calibration (see Methods).

(trace A) but less markedly than the proton uptake rate. The initial rates of H^+ uptake and of H^- transfer from a number of experiments using chromatophores from strain RTB2 are shown in Table 2.

The rate of the proton leak through the chromatophore membrane was estimated as described [14]. The procedure relies on the assumption that, following flash excitation, the burst of photosynthetic electron transport and accompanying H^+ uptake persist for only a few milliseconds. Thus, after a series of short flashes, the rate of proton efflux from the chromatophores was plotted as a function of the change in the external pH. For an equivalent change in the external pH, the proton leak is expected to be similar during light-driven and transhydrogenase-driven H^+ uptake. As was found for wild-type chromatophores, the proton leak of RTB2 membranes at the onset of the transhydrogenation reaction, calculated on this basis (but see Discussion), was only about 10% of the initial rate of proton uptake. Results for the preparations used in the measurement of the H^+/H^- ratios in the over-expressing strains are shown in Table 2.

In agreement with earlier results [14], the H^+/H^- ratio of wild-type *Rhs. rubrum* membranes was approximately 0.6 (data not shown) but, surprisingly, the H^+/H^- ratio for membranes from the over-expressing strain RTB2 was significantly greater, routinely in the region of 1.0 (Table 2). The table also shows the results of a series of experiments with RTB2 and RTB3 chromatophores performed at pH 6.3, using Chlorophenol red as a pH indicator. Again the results consistently yielded an H^+/H^- ratio of approximately 1.0. Because the rate of transhydrogenation is much lower at this pH, the signal/noise was rather poor and it was not possible to obtain the H^+/H^- ratio for wild-type chromatophores under these conditions.

3.3. Measurement of the H^+/H^- ratio, (b) in proteoliposomes prepared with transhydrogenase from beef-heart mitochondria

Mitochondrial transhydrogenase was incorporated into liposomes in the presence of Triton X-100 and the detergent was subsequently removed by adsorption on to Bio-Beads (see Methods). The method derives from that developed by Rigaud and colleagues [24], who have described procedures in which various membrane proteins have been incorporated into reproducibly well-defined liposomes. The transhydrogenase liposomes obtained in this way were well-coupled. Routinely, $AcPdAD^+$ reduction by NADPH was stimulated by 15- to 20-fold after addition of 1.5 μM FCCP in a medium of 50 mM choline chloride, 1 mM Tricine-KOH (pH 7.0), which compares favorably with a 4- to 5-fold stimulation for proteoliposomes prepared by cholate dialysis, as described in previous experiments [6–9]. The apparent decrease in specific activity of the enzyme after reconstitution is only a consequence of the different assay conditions; beef-heart transhydrogenase is highly sensitive to the ionic environment and detergent/phospholipid composition as recently discussed [23].

The results of experiments to measure the H^+/H^- ratio in mitochondrial transhydrogenase are shown in Fig. 3. To optimize and prolong the initial rate of proton uptake, it was found to be advantageous to use K^+-loaded liposomes diluted into a K^+-free medium and to treat with valinomycin just a few seconds before initiating the transhydrogenase reaction. This procedure will have led to a negative-inside diffusion potential across the liposome membrane to favour the proton translocation reaction. A similar strategy was used by Fisher and colleagues [6–9]. The character of the proton uptake and transhydrogenation reactions were similar to those observed for the chromatophore suspensions. Following the artefactual drop in pH upon $AcPdAD^+$ addition, the liposomes took up protons at an initially high rate which declined over a period of several seconds (trace B). The rate of transhydrogenation (trace A) declined less dramatically. Control experiments showed that membrane-impermeant buffer (trace C), omission of NADPH (trace D) or inclusion of nigericin (trace E), all abolished the increase in Phenol red absorbance

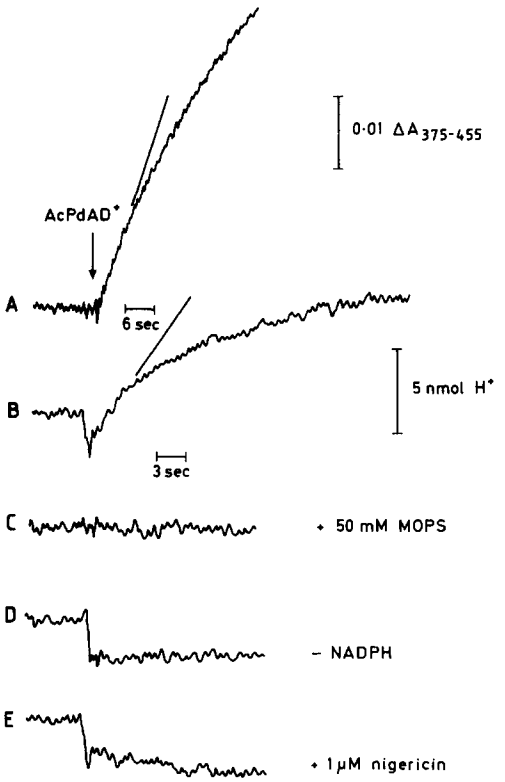

Fig. 3. The kinetics of transhydrogenation and of proton uptake by proteoliposomes containing transhydrogenase purified from beef-heart mitochondria. Assays were performed in 1 mM Tricine, 50 mM choline chloride (pH 7.0) (TCB medium). The 1 × 1 cm cuvette contained 2.5 ml TCB medium plus 0.5 ml of the proteoliposome preparation (see Methods), 100 μM NADPH, 60 μM Phenol red, 2.5 μg ml^{-1} valinomycin. After equilibration, 100 μM $AcPdAD^+$ was added where indicated. Trace A shows the reduction of $AcPdAD^+$ measured at 375–455 nm. Traces B–E show absorbance changes at 560–640 nm. Traces C and E were taken from experiments in which the medium was supplemented with either MOPS or nigericin, as shown. Trace D was recorded in the absence of NADPH in the assay mixture.

upon addition of $AcPdAD^+$. These results establish that all the observed absorbance changes of the Phenol red were a result of external pH changes, that the disappearance of protons is a direct consequence of transhydrogenation, and that proton disappearance is only a result of translocation of the ion. It is evident from the data in Fig. 3B, even with the improved time resolution provided by the indicator technique, that there is significant error on the measurement of the initial rate of proton uptake. However, it was clear from repeat experiments (not shown) that the best construct made by visual inspection (see Fig. 3B) was highly reproducible.

Pairs of values of rates of H^+ uptake and of H^- transfer for two different preparations of liposomes inlaid with mitochondrial transhydrogenase are shown in Table 2. The results repeatedly gave H^+/H^- ratios in the region of 1.0. The mean value was 1.0 ± 0.1.

4. Discussion

4.1. Measurement of H^+/H^- ratio

The procedure that we have employed for the determination of the H^+/H^- ratio (this paper and [14]) is similar to that used by Fisher and colleagues [6–9]. There are, however, two major advantages in our current method. The replacement of a glass electrode by a pH indicator dye, together with application of a rapid mixing device, enabled us to improve the time resolution of measurement by an order of magnitude as compared to data in [6–9].

Using proteoliposomes inlaid with transhydrogenase purified from beef-heart mitochondria the H^+/H^- ratio was found to be 1.0 (Table 2). This is in agreement with the conclusion of Refs. [6–9], but in our experiments an extrapolation to zero time was not required – initial rates were measured directly. The importance of the time resolution is evident from Fig. 3: true initial rates could be measured only within the first 3 s of the start of the reaction. It is evident from Fig. 3 that proton uptake measurements made 10 s after the initiation of reaction (as in Refs. [6–9]) will be a serious underestimate of the initial rate. This, and the limitations imposed by the response time of the glass electrode, at least in part, account for the spread of results in the earlier work (H^+/H^- ratios from 0.35 to 0.84 [6–9]). While the slow response of the glass electrode in those experiments would tend to decrease the estimate of the H^+/H^- ratio, the use of only the valinomycin-stimulated component of the transhydrogenation rate (which, for reasons given [13], we believe is not justified) would have inflated the apparent value of the ratio.

If the H^+/H^- ratio is indeed 1.0, a more complete description of the results of Eytan et al. [10] can now be presented. In proteoliposomes reconstituted with both transhydrogenase and F_0F_1-ATPase, it was found, at low concentrations of ATP, and after making corrections for preparation inhomogeneity (non-coupled transhydrogenase and oligomycin-insensitive ATPase), that about three transhydrogenation reactions were driven per ATP hydrolysed. Thus, assuming 4.0 H^+ translocated per ATP hydrolysed [15], the activity ratio would have resulted if 75% of the ATP-generated proton flux proceeded through transhydrogenase and 25% through non-specific leaks.

In view of structural and mechanistic similarities (see reviews [1–3]), the H^+/H^- ratio is expected to be the same in transhydrogenases from different sources. On this basis, the finding that the ratio has a value of 1.0 also in chromatophores from the transconjugant strains of *Rhs. rubrum*, RTB2 and RTB3, is reassuring (Table 2). However, it is not clear why the wild-type chromatophores, expressing lower levels of the enzyme, should give significantly smaller values of the H^+/H^- ratio. Though explanations can be considered as to why some of the earlier results might have been underestimated, namely a failure to reach equilbrium [12], difficulties in calibration [11,13]

and experimental imprecision [11] – the possible problems were discussed at length in the original articles, other data, notably those reported [14], are not so easily explained away. It is highly unlikely that the measurement of the transhydrogenation rate is in error, and so we must explain why the measured rate of proton uptake is an underestimate. The notion that the proton uptake rate is underestimated because the chromatophore membranes leak protons was rejected [14] because the rate of the leak was measured independently and shown to be insignificant.

To account for the discrepancy in the H^+/H^- ratios, we propose that the distribution of transhydrogenase in the membrane vesicles of the 'chromatophores' is not homogeneous. In wild-type bacteria it is suggested that a substantial fraction of the enzyme is located in regions of the membranes in which the quantity of the photosynthetic electron transport components is low (e.g., in the cytoplasmic membrane where the respiratory electron transport components are thought to predominate [31]), and that the proton leak of the vesicles derived from these regions is relatively high. The existence of this leak leads to an underestimate of the H^+ uptake rate. The proton leak rate (see Methods and [14]) is only measured in those vesicles with a large content of photosynthetic electron transport components (ie. those derived from the intracytoplasmic membrane), and therefore the correction is inadequate. In the transconjugant *Rhs. rubrum* strains that over-express transhydrogenase, the enzyme might be assembled, either more randomly within the membranes of the bacteria (where the leak is lower), or within localised regions of the membrane with a lower proton permeability. Both of these would give rise to a more reliable H^+/H^- ratio, but the latter possibility is somewhat less likely because in 'chromatophores' from the transconjugant strains light-driven transhydrogenation proceeds at a high rate. The heterogeneous nature of the membrane system in photosynthetic bacteria was reviewed [31].

Despite this one remaining uncertainty over wild-type chromatophores, it seems reasonable to conclude, from the data on chromatophores from the over-expressing strains of *Rhs. rubrum,* and on proteoliposomes containing mitochondrial transhydrogenase, described above, that the mechanistic H^+/H^- ratio is 1.0. Earlier results from a number of laboratories [6–14], though sometimes indirect, or limited by experimental accuracy, are clustered in the range 0.3–1.0, and are probably consistent with a value of 1.0.

4.2. Possible implications for the mechanism of proton-translocating transhydrogenase

Plausible models for transhydrogenase involving either direct or indirect coupling of transhydrogenation to proton translocation have been reviewed and discussed [1–3]. A whole-number stoichiometry of one proton translocated per hydride ion transferred is consistent with a number of possibilities. In principle, the notion of 'slip' (hydride transfer without proton translocation), can be accommodated in both direct and indirect models for coupling (discussed in [14]) but, in view of the reliability and precision of existing data, it is not a serious consideration at the present time. Note that the H^+/H^- ratios in chromatophores from the over-expressing strains of *Rhs. rubrum* were similar at pH 6.3 and at pH 7.3 (Table 2) giving no indication of any pH dependence of a slip reaction, at least within the measured range.

Recent experiments with *E. coli* and mitochondrial transhydrogenase have shown that binding/release of NADP(H) to/from the enzyme is accompanied by binding/release of a proton; there is good reason to consider that this is the translocated proton [23,32,33]. As discussed in [23,32,33], the most likely mechanism of such proton binding/release is a change in the pK_a of a group on the enzyme in response to NADP(H) binding/release; translocation is effected by a subsequent change in accessibility of the proton binding group from one aqueous phase to the other. This represents 'indirect' conformational coupling and is fully consistent with an $H^+/^-$ ratio of 1.0.

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