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Reaction between the soluble and membrane-associated proteins of the H^+-transhydrogenase of *Rhodospirillum rubrum*

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The soluble component (Th_s) was removed from the H^+-transhydrogenase of membranes from *Rhodospirillum rubrum* by washing. The depleted membranes (C_T-particles), and Th_s, separately, had no transhydrogenase activity. Re-assembly of active enzyme from Th_s and C_T-particles was followed by recording the rate of the transhydrogenase reaction as a function of time, after mixing. In the absence of nicotinamide nucleotides the formation of active enzyme was slow ($t_{1/2} > 60$ s). $NADP^+$, NADPH, to a lesser extent, NADH, but not NAD^+, increased the rate of re-assembly of active H^+-transhydrogenase. In the presence of 20 μM each of $NADP^+$ and NADH the formation of active enzyme proceeded with a $t_{1/2}$ of approx. 5 s. The presence of a proton electrochemical gradient during re-assembly had no effect on the re-assembly kinetics. The nucleotides were only effective in promoting re-assembly of active enzyme if they were present after the formation of an encounter complex between Th_s and the C_T-particles.

Introduction

H^+-Thase is a proton pump (for reviews, see Refs. 1,2). It couples the transfer of H^- equivalents between NAD(H) and NADP(H) to the translocation of H^+ across membranes:

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

In mitochondrial and bacterial membranes the enzyme probably operates from left to right under most physiological conditions. Thus, it consumes the proton electrochemical gradient generated by respiratory (or photosynthetic) electron transport to drive the synthesis of NADPH from NADH.

Mitochondrial H^+-Thase is a dimer of two identical polypeptides [3,4]. The M_r of each polypeptide is 109000. The enzyme from *Escherichia coli* is an α_2-β_2 tetramer [5]. When the α (M_r 54000) and β (M_r 49000) polypeptides are arranged contiguously there is strong homology with the mitochondrial polypeptide [3,6]. The amino acid sequence and the requirement for detergent for solubilisation of both these proteins clearly indicate that the polypeptides of both enzymes are membrane-bound. H^+-Thase from the photosynthetic bacterium, *Rhodobacter capsulatus*, has two polypeptides (M_r, 53000 and 48000) and also requires detergent for solubilisation [18]. However, the enzyme from another photosynthetic bacterium, *Rhodospirillum rubrum*, is different.

Fisher and colleagues [7–13] showed that the H^+-Thase activity of membrane vesicles (chromatophores) from *Rhs. rubrum* was lost after centrifugation in the absence of detergent through low ionic strength buffer but was regained after addition of an extract of the soluble (supernatant) fraction to the membranes. We have recently purified the soluble component. It comprises a single polypeptide, M_r 43000 on SDS PAGE, but might be dimeric under non-denaturing conditions [15]. The N-terminal amino acid sequence has strong homology with both the mitochondrial H^+-Thase and the α polypeptide of the enzyme from *E. coli*. It was concluded that this 43 kDa protein (called Th_s) is equivalent to the relatively hydrophilic domain (approx. 400 residues) at the N-terminus of mitochondrial H^+-Thase. The Th_s-depleted membranes probably contain the equivalent of the membrane-spanning domain plus the relatively hydrophilic C-terminal domain

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Abbreviations: H^+-Thase, nicotinamide nucleotide H^+-transhydrogenase; Th_s, soluble component of H^+-Thase from *Rhs. rubrum*; Th_m, membrane-bound component of the H^+-Thase of *Rhs. rubrum*; C_T-particles, chromatophores of *Rhs. rubrum* washed to remove Th_s.

of the mitochondrial enzyme. However, the polypeptide composition of this component (called Th_m) is not yet known.

There is evidence that the hydrophilic N-terminal domain of mitochondrial H^+-Thase [16,17] has a binding site for NAD(H) and that the hydrophilic C-terminal domain has an NADP(H) binding site [17] (see also Ref. 6). On the basis of the expected homologies Th_s should possess the NAD(H) site and Th_m, the NADP(H) site. Observations on protection by nucleotides against chemical modification and proteolysis suggested that Th_s has binding sites for both NAD(H) and NADP(H) and that Th_m has a binding site for NADP(H) [10,11,14].

In the study of H^+-Thase an advantage of the enzyme from *Rhs. rubrum* is that it can be separated into Th_s and Th_m, both of which have nucleotide binding sites but neither of which has catalytic activity. The separated components can be reconstituted under mild conditions to regain full H^+-Thase activity. In this report the kinetics of recombination of Th_s and Th_m are described.

Materials and Methods

Rhs. rubrum was grown and chromatophores, C_T-particles (chromatophore membranes depleted of Th_s), crude Th_s ('Type II', see Ref. 8) and purified Th_s were prepared as described [15].

Experiments were performed in 125 mM sucrose, 2.67 mM $MgCl_2$, 44 mM Tris-HCl (pH 8.0) with either chromatophores or C_T-particles at either 6.7 or 13.3 μM bacteriochlorophyll in a total volume of 3.0 ml in a 1 cm square cross-section cuvette. Transhydrogenase activity was measured as the reduction of thio-$NADP^+$ (an analogue of $NADP^+$) by NADH at 395–450 nm ($E = 10.65\ mM^{-1}\ cm^{-1}$ for thio-NADPH). The contents of the cuvette were continuously stirred with a stainless-steel blade driven by an overhead motor. The speed of the motor was adjusted to give rapid mixing of the sample while maintaining minimum optical disturbance. The mixing time was measured from the kinetics of the absorbance change resulting from the addition of a solution of NADH in the absence of membranes. Routinely, the time taken to reach 90% of the final absorbance level was approx. 0.4 s. Photosynthetic illumination, saturating with respect to transhydrogenase activity, was provided with a light-emitting diode [19].

Results

The rate of light-driven transhydrogenase of chromatophores of *Rhs. rubrum* was depleted, typically by 90%, after centrifugation through Tris buffer [7,15]. The activity of the washed membranes (called-C_T particles) was restored by addition of crude Th_s (which, itself, had no transhydrogenase activity). For each set of experiments, preliminary titrations were carried out to determine the quantity of Th_s required to give 50–80% recovery of the steady-state rate.

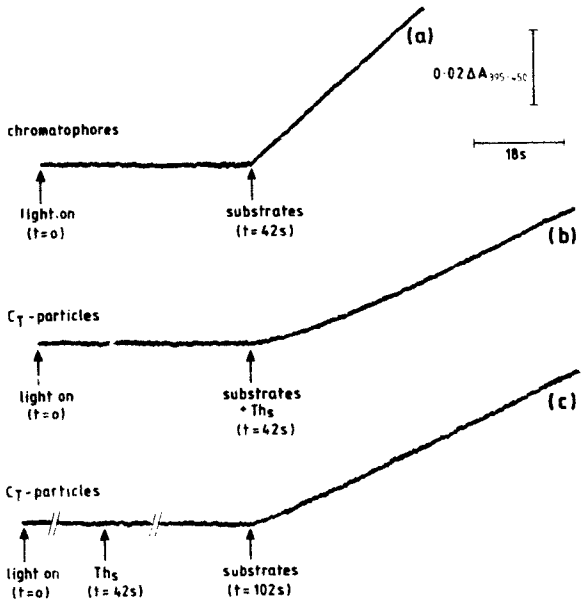

Fig. 1. The rate of formation of active H^+-Thase upon mixing Th_s with C_T-particles in the absence of added nucleotides. (a) Chromatophores (6.7 μM bacteriochlorophyll) were incubated at 30°C in the medium described in the Materials and Methods. At $t = 0$ the photosynthetic light was switched on and left on for the rest of the experiment. A mixture of the substrates, NADH and thio-$NADP^+$ (final concentrations 133 and 33 μM, respectively) was added at $t = 42$ s, as shown. (b) C_T-particles (6.7 μM bacteriochlorophyll) were incubated at 30°C in the medium described in the Materials and Methods. At $t = 0$ the photosynthetic light was switched on and left on for the rest of the experiment. At $t = 42$ s, as shown, 0.5 mg 'Type II' Th_s was added, simultaneously with a mixture of substrates, NADH and thio-$NADP^+$ (final concentrations 133 and 33 μM, respectively). (c) As in (b), except that Th_s was added at $t = 42$ s and the nucleotide substrates were added at $t = 102$ s, as shown. In all cases the rapid (artifactual) change due to the NADH was subtracted from the traces.

Fig. 1a shows that the addition of nucleotide substrates (thio-$NADP^+$ and NADH) to an illuminated suspension of chromatophores of *Rhs. rubrum* led to a prompt start of the transhydrogenase reaction at its maximum rate. In contrast, Fig. 1b shows that, after adding Th_s and a mixture of nucleotide substrates simultaneously to illuminated C_T-particles, there was a lag period of a few seconds before the onset of the maximum rate of transhydrogenase. Evidently, re-assembly of the active complex of H^+-Thase from Th_s and Th_m (in the C_T-particles) is slow relative to the catalytic turnover of the complete enzyme. The kinetics of re-assembly are described by the dependence of the

rate of the transhydrogenase reaction on the time after mixing (Fig. 2).

When the illuminated C_T-particles were incubated with Th_s for 60 s before addition of the nucleotide substrates, Fig. 1c, the lag was decreased but not eliminated. This indicates that, in the absence of nucleotides, the rate of formation of the active complex of H^+-Thase is extremely slow; only after addition of the nucleotide substrates does its rate of formation reach that described by Fig. 2.

The conclusion that the presence of nucleotides leads to a more rapid rate of formation of active enzyme is supported by Fig. 3. Here illuminated C_T-particles were pre-incubated with low concentrations (20 μM) of $NADP^+$ (the physiological substrate, not the analogue) and NADH. Then, a combination of thio-$NADP^+$ and NADH (at higher concentrations) was added: (a) simultaneously with Th_s or; (b) 10 s or; (c) 30 s after the Th_s. Note that the $NADP^+$ and NADH will act as substrates for the H^+-Thase (K_m values: 33 μM and 8.3 μM, respectively [20]) but that the reaction is not detected by spectroscopy. As in Fig. 1b formation of the active transhydrogenase complex was not instantaneous when Th_s was added at the same time as the mixture of thio-$NADP^+$ and NADH – transhydrogenase activity only developed after a lag (Fig. 3a). However, in contrast to the situation in the absence of nucleotides (Fig. 1c), the lag following the addition of thio-$NADP^+$ and NADH was much diminished after 10 s pre-incubation, and completely eliminated after 30 s pre-incubation of C_T-particles with Th_s in the presence of $NADP^+$ and NADH (Figs. 3b and c, respectively).

Fig. 4 shows complementary experiments to measure the rate of formation of active H^+-Thase from C_T-particles and Th_s in the absence (closed symbols) and presence (open symbols) of nucleotides (20 μM $NADP^+$ and NADH). As an index of the extent to which the active complex was formed after a certain

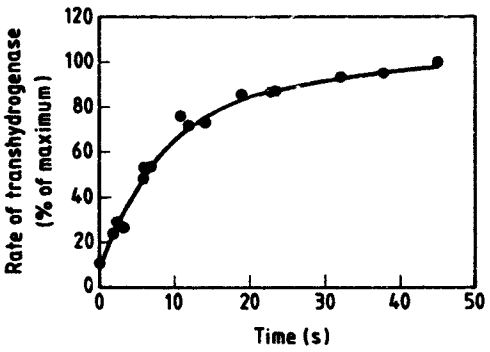

Fig. 2. The dependence of the transhydrogenase rate upon time after simultaneously mixing Th_s and nucleotide substrates with C_T-particles. The data are replotted from Fig. 1b.

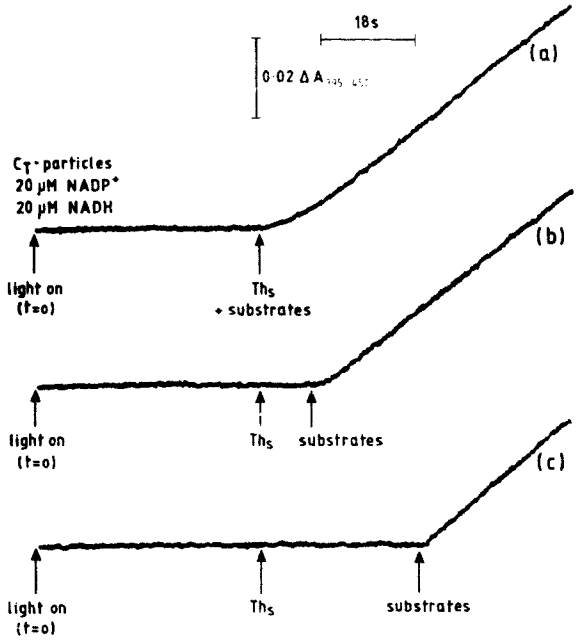

Fig. 3. The rate of formation of active H^+-Thase upon mixing Th_s with C_T-particles in the presence of added nucleotides (a) As described in Fig. 1b, except that 20 μM $NADP^+$ and 20 μM NADH were present throughout the experiment. (b) As in (a), except that the Th_s was added at t = 42 s and the mixture of thio-$NADP^+$ and NADH was added at t = 52 s. (c) As in (b) except that the thio-$NADP^+$ and NADH were added at t = 72 s.

period of incubation of C_T-particles with Th_s, we used the rate of transhydrogenase 3 s after the addition of substrates (thio-$NADP^+$ and NADH) expressed as a percentage of the ultimate steady-state rate. Note that the steady-state rate of reduction of thio-$NADP^+$ was not affected by either the time of incubation of C_T-particles with Th_s or by the presence or absence of the 20 μM $NADP^+$ and NADH. When the incubation (of C_T-particles with Th_s) was carried out in the presence of nucleotides the index approached 100% (i.e., formation of active H^+-Thase was complete) in approx. 30 s. Importantly, the dependence of the extent of active complex formation upon the time of incubation of C_T-particles with Th_s in the presence of nucleotides, estimated by this procedure was similar to the kinetics of re-assembly shown in Fig. 2. In the absence of nucleotides the dependence of active complex formation upon the time of incubation of C_T-particles with Th_s was considerably slower and was not complete within 60 s. The most typical data are shown in Fig. 4, although in the absence of added nucleotides, there was substantial variation between preparations, possibly a result of different levels of nucleotide contamination in the membranes from the original cellular material.

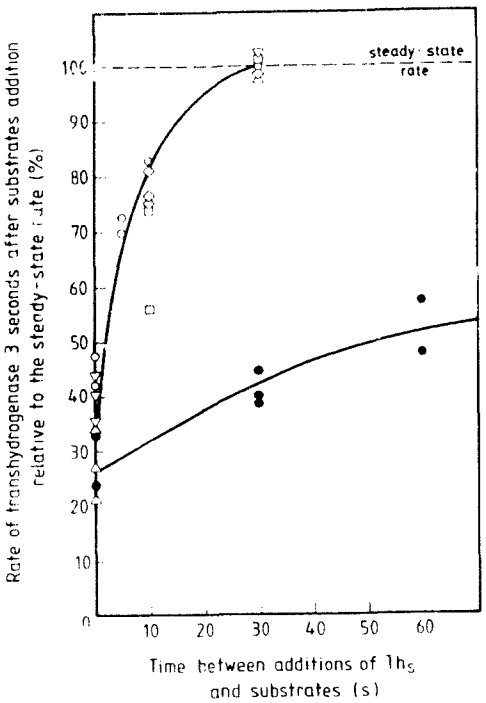

Fig. 4. The rates of formation of active H^+-Thase upon mixing Th_s with C_T-particles in the absence and presence of nucleotides. Closed symbols, absence of nucleotides; experiments were performed, as described in Fig. 1c, except that the time between the addition of Th_s and the mixture of thio-$NADP^+$ and NADH was varied, as shown. Open symbols, presence of nucleotides (20 μM $NADP^+$ and 20 μM NADH); experiments were performed as described in Fig. 3b, except that the time between the addition of the mixture of Th_s and the thio-$NADP^+$ and NADH was varied, as shown. The different sets of open symbols represent experiments with different preparations. In both sets of experiments the rate of transhydrogenase 3 s after addition of thio-$NADP^+$ and NADH as a percentage of the ultimate maximum rate (approx. 40 s later) is plotted on the ordinate.

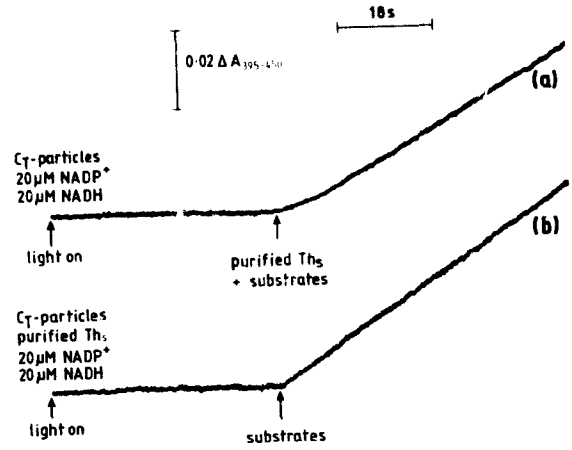

Fig. 5. The rate of formation of active H^+-Thase upon mixing pure Th_s with C_T-particles in the presence of added nucleotides. The experiment was carried out as described in Fig. 3 except that, 1.5 μg purified Th_s was used.

In most of the experiments described in this report crude Th_s was used. Only small amounts of purified protein can be prepared from manageable quantities of bacterial cells [15]. Where equivalent experiments were carried out, similar data were obtained with both crude and purified material. Fig. 5 shows the results of an experiment with purified Th_s that are analogous to those in Fig. 3a and c. Also, with pure protein, the lag in the onset of the transhydrogenase reaction, corresponding to re-assembly of the active complex (Fig. 5a), was abolished after 30 s incubation of C_T-particles with Th_s in the presence of nucleotides (Fig. 5b).

It was of interest to discover whether the transmembrane proton electrochemical gradient (Δp) affected the rate of formation of the active H^+-Thase upon

TABLE I

Nucleotides exert their effect on the rate of formation of active H^+-Thase complex only after formation of an encounter complex between Th_s and Th_q

All incubations were at 30°C. Illumination of C_T-particles was started at $t = 0$ and continued throughout each experiment. $NADP^+$ and NADH (both at 20 μM) were added to the suspension of C_T-particles just before illumination in experiments b, c and e but not in experiments a and d. The TH_s was pre-incubated with $NADP^+$ and NADH (both at 20 μM) for 180 s in experiments d and e but not in experiments a, b and c. In all cases Th_s was added to the C_T-particles at $t = 42$ s. The mixture of thio-$NADP^+$ and NADH was added either at the same time as the Th_s (experiments a, c, d and e) or 30 s later (experiment b). Note, therefore, that experiments a and b were equivalent to those shown in Figs. 1b and 3c, respectively. The 'lag index' is the rate of transhydrogenase 3 s after addition of the thio-$NADP^+$ and NADH relative to the ultimate steady-state rate. The figures give the mean value ± S.D., Number of measurements in parentheses.

Experiment	Nucleotides present during pre-incubation of C_T-particles	Nucleotides present during pre-incubation of Th_s	Time of addition of 33 μM thio-$NADP^+$ and 133 μM NADH(s)	Lag index (%)
a	none	none	42	26 ± 6 (3)
b	$NADP^+$ and NADH	none	72	100 ± 0 (3)
c	$NADP^+$ and NADH	none	42	28 ± 6 (3)
d	none	$NADP^+$ and NADH	42	32 ± 4 (3)
e	$NADP^+$ and NADH	$NADP^+$ and NADH	42	45 ± 7 (3)

mixing Th_s and C_T-particles. Since C_T-particles are capable of high rates of ATP synthesis [7], it is assumed that removal of Th_s from membranes does not substantially raise the proton conductance. The experiments described above were all performed at high Δp with illuminated membranes to maintain a large activity of the enzyme. In the dark the rate of transhydrogenase is about 1/10th of that in the light and under these conditions the signal/noise ratio was not good enough to give reliable information on the early kinetics of reaction. In order to examine the rate of formation of active complex at $\Delta p = 0$, the following strategy was adopted. A control experiment was performed during continuous illumination, as described in Fig. 3b (see above). In a second experiment dark conditions were maintained initially but the photosynthetic light was switched on at the same time that the nucleotide substrates were added. Thus, in this case Th_s was allowed to interact with C_T-particles (in the presence of 20 μM $NADP^+$ and NADH) for 10 s in the absence of a proton electrochemical gradient before driving the reaction at a high rate with an elevated Δp. Though there was some distortion of the transhydrogenase kinetics due to light-induced pigment absorbance changes, it was concluded that the lag at the onset of the reaction was similar to that in the control (data not shown). Apparently, Δp does not have a significant effect on the re-assembly of the complex.

The accelerative effect that nucleotides have on the rate of formation of the active complex of H^+-Thase could, in principle, arise as a consequence of their binding to either Th_s or Th_m before mixing or it might be due to the facilitation of conformational changes following the production of an encounter complex of Th_s and Th_m. The experiments in Table I distinguish between these possibilities. As above, low concentrations of $NADP^+$ and NADH were used to promote activation in the absence of detectable transhydrogenase reaction. Thio-$NADP^+$ and NADH (at higher concentrations) were used to monitor the transhydrogenase rate and, from the initial kinetics, to provide an index of the active state of the enzyme. In (a), a control, equivalent to Fig. 1b, the 'lag index' of 26% was characteristic of slow re-assembly. In (b), a control, equivalent to Fig. 3c, the lag was eliminated (lag index = 100%) when the $NADP^+$ and NADH were pre-incubated with the C_T-particles for 60 s and were present during the combined incubation of C_T-particles and Th_s (for 30 s) before the addition of thio-$NADP^+$ and NADH. However, when in (c) the $NADP^+$ and NADH were only present during the pre-incubation period with C_T-particles, the lag (index = 28%) was similar to that in (a). In a complementary experiment (d), Th_s was pre-incubated for 180 s with $NADP^+$ and NADH but the lag was still pronounced (index = 32%) after adding this mixture simultaneously with the thio-$NADP^+$ and NADH to C_T-particles. Experiment (e) shows that even when Th_s and C_T-particles were separately incubated with $NADP^+$ and NADH before being mixed simultaneously with thio-$NADP^+$ and NADH, there was still a considerable lag (index = 45%). These data show that the effect of nucleotides in promoting the formation of active complex is exerted only after formation of an encounter complex between Th_s and Th_m. The nucleotides do not have any effect on the separate components.

TABLE II

The effects of nicotinamide nucleotides on the re-assembly of active H$^+$-Thase from Th_s and C_T-particles

C_T-particles (13.3 μM bacteriochlorophyll) were incubated at 30°C in the medium described in the Materials and Methods in the presence of the nucleotide shown in the table (all at 20 μM). At $t = 0$, the photosynthetic light was switched on and left on for the rest of the experiment. At $t = 42$ s, 0.5 mg 'type II' Th_s was added and, at $t = 52$ s, 133 μM NADH and thio-$NADP^+$ were added. The transhydrogenase rate was measured 6 s later and expressed as a percentage of the ultimate maximum rate (approx. 40 s later). The figures give the mean value ± S.D. Number of measurements in parentheses.

Nucleotide	Transhydrogenase rate 6 s after mixing with substrates, as percentage of the ultimate maximum rate
None	54 ± 4 (3)
$NADP^+$	77 ± 1 (3)
NADPH	78 ± 3 (3)
NADH	68 ± 5 (3)
NAD^+	55 ± 3 (3)
$NADP^+$ + NADH	89 ± 2 (3)
$NADP^+$ + NADPH	73 ± 6 (3)
$NADP^+$ + NAD^+	74 ± 8 (3)
NADPH + NADH	81 ± 2 (3)
NADPH + NAD^+	72 ± 1 (3)
NADH + NAD^+	65 ± 4 (3)

Table II and Fig. 6 show the results of experiments to investigate the ability of different nucleotides, separately and in combination, to increase the rate of formation of active complexes of H^+-Thase from Th_s and Th_m. In preliminary experiments it was shown that a protocol similar to that in Fig. 3b, with 10 s incubation of Th_s and C_T-particles before addition of the probe substrates, gave rise to sensitive differences in the lag indices for different nucleotides. Thus (Table II), in the absence of added nucleotides, the index, representing a slow rate of re-assembly, was 54% and the most effective nucleotide combination yielded an index of 89%. Of individual nucleotides, $NADP^+$ and NADPH were equally effective, NADH was less effective and NAD^+ was ineffective at increasing the rate of formation of active complex. The concentration dependences of $NADP^+$, NADPH and NADH are shown in Fig. 6. The binding constant in each case was approx. 3

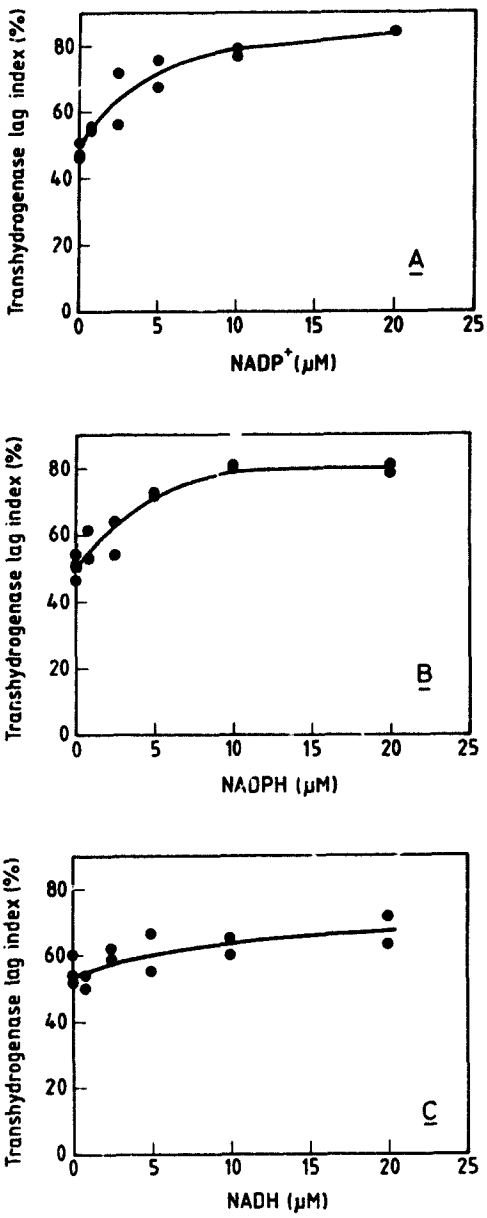

Fig. 6. The dependence of the rate of formation of active H^+-Thase upon mixing pure Th_s with C_T-particles on the concentration of added nucleotide. Experiments were performed as in Fig. 1, except that nucleotide at the concentration indicated, was present throughout and that Th_s was added at $t = 42$ s and the mixture of thio-$NADP^+$ and NADH was added at $t = 52$ s. For each experiment the 'lag index' was calculated from the rate of transhydrogenase 3 s after addition of thio-$NADP^+$ and NADH, as a percentage of the ultimate maximum rate (approx. 40 s later). A, $NADP^+$; B, NADPH; C, NADH.

μM. A combination of saturating concentrations of NADH and $NADP^+$ was more effective than the individual nucleotides (Table II), indicating that separate binding sites on the encounter complex may be involved. In contrast, a combination of saturating concentrations of $NADP^+$ and NADPH was no more effective than the individual nucleotides, consistent with the possibility that these nucleotides bind at the same site. The presence of NAD^+ seemed not to affect the ability of other nucleotides to promote formation of active complex. In separate experiments (not shown) it was found that even 30 μM NAD^+ did not affect the re-assembly kinetics in the presence of 10 μM NADH. Thus, it was not possible to decide whether NAD^+ failed to bind to the enzyme or whether it bound but had no effect on the rate of formation of active complex. Higher concentrations of NAD^+ could not be used in these experiments because they gave rise to product inhibition of the transhydrogenase reaction.

Discussion

In this report we have described the kinetics of re-assembly of the H^+-Thase of *Rhs. rubrum* from its resolved components, Th_s, a water-soluble protein, and Th_m, a membrane-bound protein, located in C_T-particles. Fig. 7 shows a minimal model to account for our findings. It is assumed that on addition of Th_s to C_T-particles an inactive encounter complex (Th_s-Th_m) is rapidly formed. It must undergo conformational rearrangement before it becomes capable of catalysis. In the absence of nucleotides (top line), conversion to the active form, Th_s-Th_m^*, is slow (Fig. 4, closed symbols). It is likely that the equilibrium does not lie far over to the right since, under these conditions, Th_s is readily dissociated from the membranes by centrifugation [7,15]. In the presence of nucleotides (bottom line), conversion to the active form is more rapid (Fig. 4, open symbols) and the equilibrium constant for binding of Th_s to the membranes may be larger (although its value probably depends on which nucleotide is present, see below).

To explain the essential observations on a quantitative basis it is assumed that rates of formation of the encounter complexes (reactions 1 and 3) and all nucleotide binding reactions (5–8) are rapid ($t_{1/2} < 1$ s) and that the $t_{1/2}$ to reach equilibrium between encounter complex and activated complex is > 60 s in the absence of nucleotide (reaction 2) and approx. 5 s in the presence of $NADP^+$ and NADH (reaction 4). Thus, in Fig. 1b, following simultaneous addition of Th_s, thio-$NADP^+$ and NADH to C_T-particles, re-assembly proceeded through the two pathways, $1 \rightarrow 7 \rightarrow 4$ and $5/6 \rightarrow 3 \rightarrow 4$ with an overall $t_{1/2}$ of approx. 5 s for production of Th_s-Th_m^*. The same pathways operated in Fig. 3a – pre-incubation of C_T-particles with $NADP^+$ and NADH influenced only reaction 6, without any effect on the kinetics of formation of active complex. However, when Th_s was incubated with C_T-particles in

the absence of nucleotides even for as long as 60 s (Fig. 1c), formation of Th_s-Th_m^* through pathway 1 → 2 was incomplete (the reaction is slow and equilibrium does not lie far to the right). On addition of thio-$NADP^+$ and NADH, Th_s-Th_m^* already present was immediately able to perform catalysis but the inactive Th_s-Th_m was converted into the active form via 7 → 4: the lag was decreased in extent but still had a $t_{1/2}$ of approx. 5 s. In contrast, when this experiment was carried out in the presence of low concentrations of $NADP^+$ and NADH (Fig. 3c), the formation of Th_s-Th_m^* was essentially complete within 30 s ($t_{1/2} \simeq 5$ s, Fig. 4) and so, on addition of thio-$NADP^+$ and NADH, the transhydrogenase reaction began promptly.

NADP(H) enhanced the sensitivity of Th_m in C_T-particles to inhibition by trypsin and protected Th_m in C_T-particles from inactivation by heat, butanedione and pentanedione [11,14], indicating that $NADP^+$ and NADPH bind to Th_m in the absence of Th_s and cause changes in protein conformation (hence inclusion of reaction 6 in Fig. 7). However, these conformational changes [11,14] must be distinct from those involved in production of active H^+-Thase since it was established (Table I) that the latter take place only after formation of an encounter complex with Th_s. There is also evidence [14] that, in the absence of Th_m, Th_s can bind NAD^+ and, surprisingly in view of more recent work [16,17], $NADP^+$. Hence, reaction 5 is included in Fig. 7.

Reaction 4 can proceed with different nucleotides bound to the encounter complex. Thus, even when only single nucleotides (such as $NADP^+$ or NADPH or NADH) are present, the formation of active complex is faster than in the absence of nucleotides (Table II and Fig. 6). The approx. $t_{1/2}$ of 5 s for reaction 4 (Figs. 2 and 4) only applies to the situation in the presence of both $NADP^+$ (or thio-$NADP^+$) and NADH.

The ultra-centrifuge experiments of Fisher and Guillory [8] revealed that the ability of C_T particles to bind Th_s was influenced by the presence of nucleotide. NAD^+ had no effect on the binding equilibrium, in agreement with our observations on the reaction kinetics. Also, low concentrations of $NADP^+$ and NADPH increased the extent of binding [8], consistent with the finding that these nucleotides both accelerated the rate of formation of active complex. However, the observations that higher concentrations of NADPH promoted dissociation of Th_s from C_T-particles and that NADH inhibited binding supported by either $NADP^+$ or NADPH [8] were not mirrored by a decreased rate of activation. Our interpretation of the centrifugation experiments is that they reflect a complex set of reactions described in summary by reactions 3 and 4 of Fig. 7 but which, in detail, encompass sub-sets of reactions that depend upon the nucleotide(s) present. The interesting conclusion that the Th_s-Th_m complex is reversibly dissociated during the process of transhydrogenation [8] seems very unlikely in the context of the observations described in this report. Formation of active complex of H^+-Thase from isolated Th_s and Th_m (in C_T-particles) in the presence or absence of nucleotides is very slow ($t_{1/2}$ from 5 s and > 60 s, respectively) compared with the turnover of the enzyme ($t_{1/2}$ approx. 50 ms [19]), compare the kinetics of Fig. 1a and b, and, therefore cannot operate as a component reaction.

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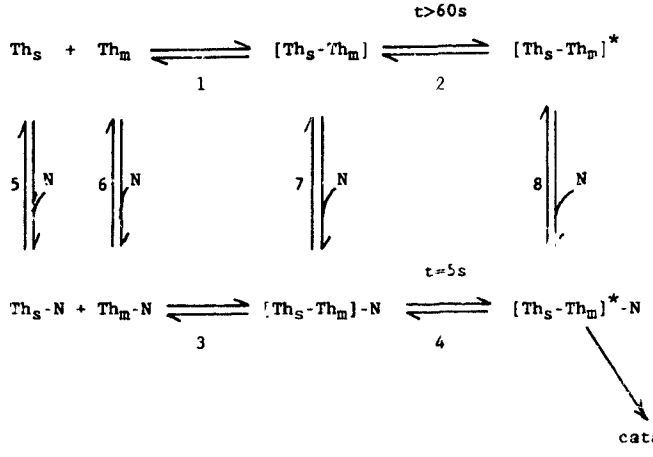

Fig. 7. A minimal model for re-assembly of active H^+-Thase from Th_s and Th_m. N, nicotinamide nucleotide, probably NAD(H) in the case of Th_s (reaction 5) and NADP(H) in the case of Th_m (reaction 6), but see text. t is the time required to reach half-maximal rate.

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