A rapid burst preceding the steady-state rate of H^+-transhydrogenase during illumination of chromatophores of *Rhodobacter capsulatus*

Implications for the mechanism of interaction between protonmotive force and enzyme

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At the onset of illumination of chromatophores there was a burst ($t_{1/2}$ approx. 5 ms) in the rate of the H^+-transhydrogenase reaction before establishment of the steady-state rate. The burst was suppressed at high pH with a pK_a of approx. 8.5. The burst and the steady-state rate were inhibited by either (i) a combination of myxothiazol and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, or (ii) NAD^+, or (iii) dicyclohexylcarbodiimide. The results support a model in which substrate binding to H^+-transhydrogenase is relatively fast. A subsequent slow step is accelerated by the protonmotive force and a third step, possibly product release, is rate-limiting in steady-state turnover during illumination.

Transhydrogenase; Photosynthetic bacteria; *Rhodobacter capsulatus*; Protonmotive force; Photosynthesis; Transient kinetics

1. INTRODUCTION

Nicotinamide nucleotide H^+-transhydrogenase (H^--Thase), found in mitochondria and widespread in bacteria, couples transfer of hydride ion equivalents between NAD(H) and NADP(H) to the protonmotive force, Δp [1].

$$NADH + NADP^+ + H^+_o \rightleftharpoons NAD^+ + NADPH + H^+_i$$

Analysis of steady-state kinetics of H^+-Thase from mitochondria and *E. coli* [2–4] and from *Rb. capsulatus* (Lever, Palmer, Cunningham, Cotton, Jackson, unpublished) established that the reaction proceeds by way of random sequential addition of the nucleotide substrates to form a ternary complex. Nucleotide binding sites have been identified in the amino acid sequence [5]. Models have been advanced to account for the coupling between H^- transfer and proton translocation [6–8] and the mechanism by which Δp might affect the rate of the reaction has been discussed [9]. Examination of pre-steady-state kinetics of enzyme-catalysed reactions, following rapid mixing with the substrates, is a useful approach to the elucidation of reaction mechanism. In this report we employed a variation on this procedure: by illuminating a previously dark suspension of chromatophores from photosynthetic bacteria, the H^+-Thase, operating initially at a slow rate, at $\Delta p = 0$, was accelerated by a rapid increase in the value of Δp.

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2. MATERIALS AND METHODS

Chromatophores of *Rb. capsulatus* were prepared [10]. Absorbance changes were recorded on a single-beam spectrophotometer (time constant 100 μs). Photosynthetic illumination was provided with an OD100 GaAlAs emitter (Opto Diode Corp.) positioned 1 mm from the cuvette and operated at 0.5 A. The emitter, which gave close to saturating rates of H^+-Thase in steady-state, was switched, in less than 10 μs. Electrochromic absorbance changes were measured at 528 nm. Reduction of thio-$NADP^+$ (Sigma) by NADH was measured at approx. 395 nm, where the extinction coefficient (corrected for absorbance decrease due to simultaneous oxidation of NADH) was 11.2 $mM^{-1} \cdot cm^{-1}$ [11]. The wavelength where light-induced absorbance transients due to chromatophore pigments were minimal, was selected by experiment for each sample of chromatophores. Transients were averaged during repeated periods of excitation. They were measured in a 10 mm^2 cross-section flow-through cuvette. After each period of excitation (duration 150 ms), when approx. 1 ml of sample was irradiated, the contents of the cuvette were pumped into a darkened reservoir, vol. 30 ml, and replaced by a fresh sample from the reservoir. Because signal-averaging was required, it was necessary to allow sufficient time between the periods of excitation to ensure that Δp was essentially zero at the start of each recording. Since Δp decays with $t_{1/2} < 5$ s upon darkening [10], then at an excitation frequency of 0.2 Hz, the level of Δp in the chromatophores in the reservoir remained $< 4\%$ of that during illumination – they were effectively de-energised. Experiments were performed under aerobic conditions at 30°C in a medium containing 10% sucrose, 30 mM KCl, 2 mM $MgCl_2$, 50 mM K^+-tricine, pH 7.6, 1 $\mu g \cdot ml^{-1}$ rotenone, 0.2 $\mu g \cdot ml^{-1}$ venturicidin. Unless otherwise stated, chromatophores (10 μM bacteriochlorophyll) were incubated in the above medium for 5 min. NADH, 267 μM, was added and the measurements were then initiated for 100 cycles. The

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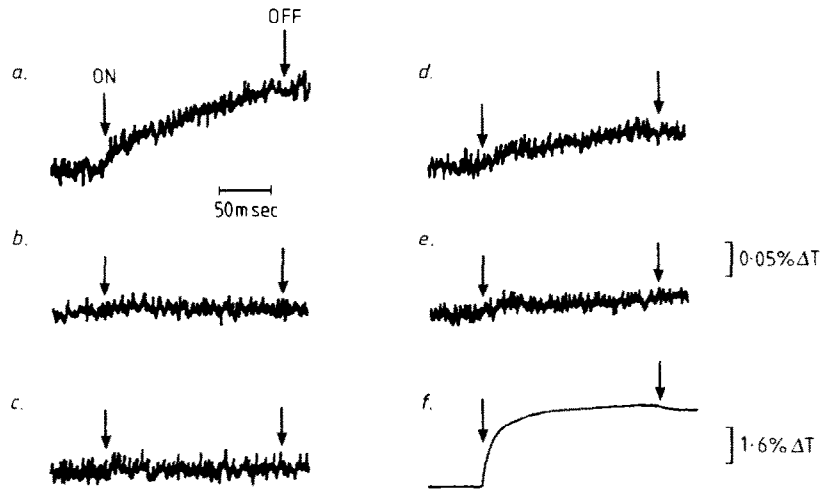

Fig. 1. Evidence for the existence of a burst in the kinetics of H^+-Thase at the onset of illumination. (a) Experiment performed as in section 2. (B) Omission of thio-$NADP^+$ from the reaction medium. (c) Replacement of thio-$NADP^+$ by 67 μM $NADP^+$ (d) Inclusion of both 67 μM thio-$NADP^+$ and 670 μM $NADP^+$ (e) As in (a) but in the presence of 5 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and 1 μM myxothiazol. (f) Light-induced electrochromic absorbance change measured under conditions similar to (a) but without signal averaging.

averaged trace, due to chromatophore pigment absorbance changes, was stored. Thio-$NADP^+$, 67 μM, was added and the measurements, averaging and data storage were repeated. The two stored traces were subtracted and the result was attributed to absorbance changes of the H^+-Thase reaction.

3. RESULTS

The kinetics of the H^+-Thase reaction at the onset of illumination, revealed by the subtraction procedure described above, are shown in Fig. 1A. Prior to reaching the steady-state there was a short burst of H^+-Thase at a much (initially 10-fold) higher rate. The half-time of the burst was approx. 5 ms and the extent was about 0.12 ± 0.04 mol/mol photosynthetic reaction centres (4 chromatophore preparations, 10 measurements). The steady-state rate continued, essentially unabated, for the next 150 ms when the light was extinguished. As discussed [9] but not shown here, the rate of H^+-Thase after illumination relaxed to the original dark rate within several hundred milliseconds.

The extent of the burst was of the same order as residual absorbance changes in the chromatophore pigments in the absence of nucleotides and considerably smaller than pigment absorbance changes at slightly longer and shorter wavelengths (not shown). The possibility that the transient seen in Fig. 1a arose artefactually either from time-dependent changes in light-induced pigment absorbance or from spectral distortion due to binding of thio-$NADP^+$ was eliminated by other experiments shown in Fig. 1. In the experiment described in Fig. 1b, thio-$NADP^+$ was not added between the two periods of recording and averaging. The subtracted data revealed that in this case there were no significant absorbance changes upon illumina-

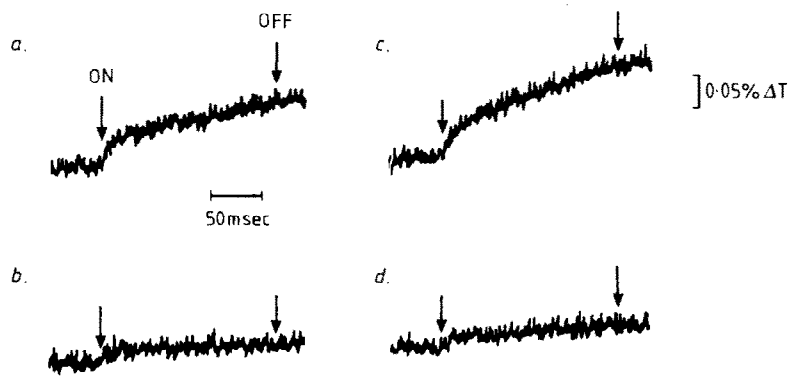

Fig. 2. Inhibition of the burst of H^+-Thase activity at the onset of illumination by NAD^+ and by DCCD. (a) Experiment performed as in section 2. (b) As in (a) but in the presence of 4 mM NAD^+. (c) and (d) Chromatophores, at 1 mM bacteriochlorophyll, were treated with either 4 μl/ml of methanol (c), or 4 μl/ml of 100 mM DCCD in methanol (d), in the dark for 1 h at room temperature. The treated chromatophore suspensions were rapidly frozen to −20°C and stored for 20 h. Experiments were then performed as in section 2.

tion. Fig. 1c shows that when $NADP^+$ was added in place of the thio-analogue, the subtracted absorbance transients at the onset of illumination were negligable (of course, there are no absorbance changes accompanying the H^+-Thase reaction with its physiological substrates). When $NADP^+$ was added together with thio-$NADP^+$, the apparent extent of the burst (relative to thio-$NADP^+$ alone) was decreased in parallel with the steady-state rate (Fig. 1d). It was noted that the presence of NADH alone (i.e. in the absence of $NADP^+$ or analogues) did lead to small differences in the light-induced absorbance change at this wavelength, perhaps through interaction with the NADH dehydrogenase.

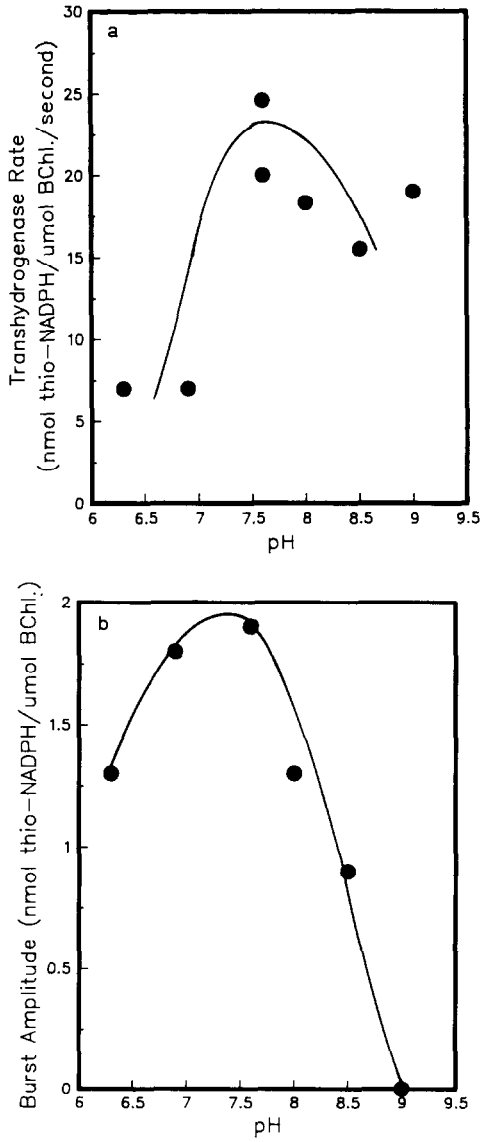

Fig. 3. pH dependence of (a) the extent of the burst of H^+-Thase activity at the onset of illumination and (b) the steady-state rate of H^+-Thase during illumination. Taken from experiments similar to those in Fig. 1a but using 50 mM Mes (pH 6.3), 50 mM Mops (pH 6.9), 50 mM tricine (pH 7.6–8.0) or 50 mM Bicine (pH 8.5–9.0).

For comparison with the H^+-Thase kinetics, Fig. 1f shows the rate of development of membrane potential ($\Delta\Psi$), the major contributor to Δp under these conditions. Upon illumination $\Delta\Psi$ rose rapidly (8 ms) to 50% of its maximum value and then more slowly. It is clear that the rate of H^+-Thase during the burst may have been limited by the build-up of $\Delta\Psi$. A combination of myxothiazol and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, which has a pronounced inhibitory effect on the development of the light-induced Δp in chromatophores [12], eliminated both the H^+-Thase burst and the steady-state rate of the reaction during illumination (Fig. 1e). These reagents have no effect on the H^+-Thase enzyme itself, as judged by their failure to inhibit the reverse reaction from NADPH to acetylpyridine adenine dinucleotide in chromatophores (not shown).

The presence of NAD^+ in the reaction medium (Fig. 2b) decreased the steady-state rate of H^+-Thase during illumination, presumably as a result of competition with NADH and formation of a dead-end ternary complex [2]. It also decreased the extent of the burst at the onset of illumination.

Treatment of the chromatophores with dicyclohexylcarbodiimide (DCCD) inhibited the steady-state rate of H^+-Thase in chromatophores and the extent of the burst at the onset of illumination (Fig. 2d). The treatment had no effect on either the rate of development or the extent of the electrochromic absorbance change during illumination (not shown), indicating that DCCD was neither inhibiting electron transport nor uncoupling the chromatophore membrane but was directly inhibiting the H^+-Thase.

Fig. 3b is in agreement with an earlier description of the pH dependence of the steady-state rate of H^+-Thase in illuminated chromatophores [9]. However Fig. 3a shows a different relationship between the extent of the H^+-Thase burst and the value of medium

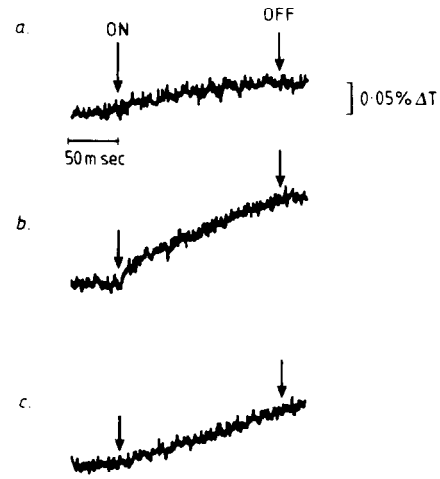

Fig. 4. The kinetics of H^+-Thase at the onset of illumination at (a) pH 6.3; (b) pH 7.6; (c) pH 9.0. See Fig. 3.

pH. At low pH, where the steady-state rate of H^+-Thase in the light was quite low, the burst was almost maximal in extent. It was, however, significantly slower than at higher pH (Fig. 4a). The extent increased slightly as the pH was raised to pH 7.5 (Fig. 4b) but further increase in the suspension pH led to a decrease in the amplitude of the burst with a pK_a at approx. 8.5. Thus, at alkaline pH, no H^+-Thase burst was evident and the steady-state was reached very soon after the onset of illumination (Fig. 4c).

4. DISCUSSION

A minimal model to explain the kinetics of H^+-Thase at the onset of illumination is as follows:

$$E + NADH + tNADP^+ \overset{K_1}{\rightleftharpoons} E \cdot NADH \cdot tNADP^+$$
$$\overset{k_2(\Delta p)}{\longrightarrow} E \cdot NAD^+ \cdot tNADPH \overset{k_3}{\longrightarrow} E + NAD^+ + tNADPH$$

The existence of a burst suggests that in the dark period before energisation, the relatively rapid addition of substrates (equilibrium constant, K_1) and the existence of a subsequent, relatively slow step (k_2) in the reaction sequence leads to the build-up of the enzyme intermediate ($E \cdot NADH \cdot tNADP^+$). The generation of Δp causes acceleration of the slow step (k_2) but then further turnover is limited by a third component (k_3). In principle, the latter could lie either after the Δp-sensitive reaction (as above) or before it. However, in our experiments we cannot distinguish between thio-NADPH which is bound to the enzyme and that which is free in solution. Thus, the burst might represent bound or free thio-NADPH. There may be spectral differences between the bound and free nucleotide but they would be difficult to detect by procedures employed here. The implication is that product (thio-NADPH) release is not the energy-dependent step. The rate-limiting step during illumination (k_3) could be the release of either NAD^+ or thio-NADPH from the enzyme. According to the model, the effect of NAD^+ in decreasing both the extent of the burst and the steady-state rate (Fig. 2a and b) can be explained by a competitive displacement of NADH from its binding site to give an unproductive, dead-end complex, $E \cdot NAD^+ \cdot tNADP^+$.

DCCD interferes with the binding of NAD(H) in mitochondrial H^+-Thase [13,14]. In the context of the model proposed here, and assuming a similar effect on H^+-Thase from chromatophores, DCCD would be expected to decrease the value of the substrate binding constant, K_1. This is consistent with its inhibitory effect on both the amplitude of the burst at the onset of illumination and on the steady-state rate during illumination (Fig. 2c and d).

The dependence on pH of the relation between the steady-state rate of H^+-Thase was recently explained with the assumption of pK_a values on the rate constants equivalent to K_2 and k_3 [9]. The data in Fig. 3a can be explained by the involvement of a proton dissociation (pK_a approx. 8.5) in one of the nucleotide binding steps making up the equilibrium described by K_1. Thus, the amplitude of the burst is decreased as K_1 decreases at alkaline pH. Although K_m^{app} values for the nucleotide substrates do not necessarily reflect the binding affinity, it is interesting that K_m^{app} for thio-$NADP^+$, in particular, but also for NADH in chromatophores, increase with pH over the range in which the extent of the burst is attenuated [9].

The maximum extent of the burst gives a limiting indication of the amount of H^+-Thase in chromatophore membranes: the enzyme must be in excess of 0.12 mol/mol reaction centres, or about 1.2 per vesicle (using data from [15]). In turn this leads to an upper limit for k_{cat} of 20 s^{-1}. A value of k_2 = 200 s^{-1} may be calculated from the initial rate of H^+-Thase at the beginning of the burst although the rate of this component is probably limited by the relatively slow rise of Δp (compare Fig. 1a with 1f).

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