ELSEVIER

Biochimica et Biophysica Acta 1231 (1995) 304–312

BBA Biochimica et Biophysica Acta

Cyclic reactions catalysed by detergent-dispersed and reconstituted transhydrogenase from beef-heart mitochondria; implications for the mechanism of proton translocation

Leonid A. Sazanov, J. Baz Jackson *

School of Biochemistry, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Received 7 March 1995; accepted 15 June 1995

Abstract

Transhydrogenase from beef-heart mitochondria was solubilised with Triton X-100 and purified by column chromatography. The detergent-dispersed enzyme catalysed the reduction of acetylpyridine adenine dinucleotide ($AcPdAD^+$) by NADH, but only in the presence of $NADP^+$. Experiments showed that this reaction was cyclic; NADP(H), whilst remaining bound to the enzyme, was alternately reduced by NADH and oxidised by $AcPdAD^+$. A period of incubation of the enzyme with NADPH at pH 6.0 led to inhibition of the simple transhydrogenation reaction between $AcPdAD^+$ and NADPH. However, after such treatment, transhydrogenase acquired the ability to catalyse the (NADPH-dependent) reduction of $AcPdAD^+$ by NADH. It is suggested that this is a similar cycle to the one described above. Evidently, the binding affinity for $NADP^+$ increases as a consequence of the inhibition process resulting from prolonged incubation with NADPH. The pH dependences of simple and cyclic transhydrogenation reactions are described. Though more complex than those in *Escherichia coli* transhydrogenase, they are consistent with the view [Hutton, M., Day, J.M., Bizouarn, T. and Jackson, J.B. (1994) Eur. J. Biochem. 219, 1041–1051] that, also in the mitochondrial enzyme, binding and release of $NADP^+$ and NADPH are accompanied by binding and release of a proton. The enzyme was successfully reconstituted into liposomes by a cholate dilution procedure. The proteoliposomes catalysed cyclic NADPH-dependent reduction of $AcPdAD^+$ by NADH only when they were tightly coupled. However, they catalysed cyclic $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH only when they were uncoupled eg. by addition of carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone. These observations are evidence that the proton binding and release which accompany $NADP^+$ binding and release, respectively, take place on the inside of the vesicle, and that they are components of the electrogenic processes of the enzyme.

Keywords: Transhydrogenase; Proton translocation; Mitochondrion; Liposome; Membrane protein

1. Introduction

Proton-translocating nicotinamide nucleotide transhydrogenase (H^+-Thase) is found in the inner mitochondrial membranes of animal cells and in the cytoplasmic membranes of bacteria (reviewed [1–3]). It couples the transfer of reducing equivalents (H^- equivalents) to the translocation of H^+.

$$NADH + NADP^+ + H^+_{out} \leftrightarrow NAD^+ + NADPH + H^+_{in} \quad (1)$$

In mitochondria, together with the NAD-and NADP-linked isocitrate dehydrogenases, H^+-Thase might serve in a substrate cycle which provides fine regulation of flux through the Krebs tricarboxylic acid cycle [4]. In bacteria its function might be in the production of NADPH for biosynthesis [5].

The primary amino acid sequences of H^+-Thase from a number of species are known [6–12]. Although the gene and polypeptide arrangements are variable (reviewed [11]), a common feature is that the enzyme seems to be composed of three large domains. Domains I and III protrude from the membrane (into the matrix in mitochondria and into the cytoplasm in bacteria) and domain II spans the membrane [13–16]. There is good evidence that H^+-Thase has separate binding sites, for NAD^+/NADH in domain I, and for $NADP^+$/NADPH in domain III [6,7,17–19]. The

Abbreviations: H^+-Thase, proton-translocating nicotinamide nucleotide transhydrogenase; $AcPdAD^+$, acetyl pyridine adenine dinucleotide; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; Δp, proton electrochemical gradient.

* Corresponding author. Fax +44 121 4143982.

SSDI 0005-2728(95)00096-8

reaction proceeds by a mechanism which involves interconversion of ternary complexes between the enzyme and the nucleotide substrates [20–23], i.e., [enzyme.NADH.$NADP^+$] ↔ [enzyme.NAD^+.NADPH]. There are no known metal centres or prosthetic groups in the enzyme. The central point of interest is the mechanism by which the transfer of H^- equivalents between the nucleotides is coupled to the translocation of H^+ across the membrane. We considered the possibility that binding and release of $NADP^+$ and NADPH to and from the enzyme are central in the energy-coupling mechanism [24,25].

In 1981 Fisher and colleagues made an interesting observation [26]: in coupled proteoliposomes inlaid with mitochondrial transhydrogenase, but not in uncoupled proteoliposomes and not with the detergent-solubilised enzyme, the rate of reduction of the NAD^+ analogue, $AcPdAD^+$, was more rapid with a combination of NADPH and NADH than with NADPH alone (the reduction of $AcPdAD^+$ by NADH alone was very low). During the reduction of $AcPdAD^+$ by NADPH *plus* NADH, the reducing equivalents were derived from the latter nucleotide. A year later, Fisher and colleagues [27], and Rydstrom and colleagues [21], independently developed a plausible explanation for these observations: they proposed a cycle in which NADPH reduces $AcPdAD^+$, accompanied by the inward pumping of protons, and then NADH reduces $NADP^+$, accompanied by the outward pumping of protons. In a review article, Fisher and Earle [27] made the specific suggestion that the development of a proton electrochemical gradient (Δp, positive inside the vesicle) might decrease the rate of release of $NADP^+$ from the enzyme and, therefore, that this step might be involved in energy coupling.

In our laboratory it was observed that, under some conditions (notably low pH and low ionic strength), this reaction, NADPH-dependent reduction of $AcPdAD^+$ by NADH, occurred in *solubilised* purified H^+-Thase from bacteria [24,25]. Discovery of the complementary process, $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH, and a detailed characterization of both of these reactions, established that they arise from a cyclic set of events in which (under appropriate conditions) NADP(H) remains permanently bound to the enzyme [24,25]. It was proposed that $AcPdAD^+$ is reduced by enzyme-bound NADPH and then NADH is oxidised by enzyme-bound $NADP^+$. The pH dependence of these and other reactions catalysed by *E. coli* H^+-Thase led to the conclusion that release of $NADP^+$ and of NADPH from the enzyme are accompanied by the release of a proton. In view of the findings of Fisher and Rydstrom and colleagues on the reconstituted mitochondrial protein (above), we suggested that the proton binding/release reactions accompanying NADP(H) binding and release might be components of proton translocation. However, the observation [21,27] that NADPH-dependent reduction of $AcPdAD^+$ by NADH does not occur with the solubilised transhydrogenase from mitochondria might be seen to compromise our general hypothesis on the mechanism of proton translocation. In this report we show that NADPH-dependent (and $NADP^+$-dependent) reduction of $AcPdAD^+$ by NADH do occur in solubilised purified H^+-Thase from beef-heart mitochondria, but only at low pH under certain conditions. It is proposed that differences with the *E. coli* enzyme arise partly from a difference in the apparent pK_a of the group which binds protons during NADP(H) binding. We confirm that NADPH-dependent reduction of $AcPdAD^+$ by NADH in proteoliposomes reconstituted with mitochondrial transhydrogenase can be observed only in the absence of an uncoupler [21,26,27], and we show that $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH in proteoliposomes has a *requirement* for uncoupler. The latter observation provides complementary evidence on the Δp dependence of nucleotide binding. Taken together, these observations support the proposed mechanism of reaction [24,25], in which those proton binding/release reactions that accompany NADP(H) binding/release are associated, in the membrane-bound enzyme, with the process of proton translocation.

2. Methods

Beef-heart mitochondria and submitochondrial particles were prepared essentially as described [28]. Earlier protocols for purification of beef-heart transhydrogenase involving detergent extraction, either with Triton X-100 [32] or cholate [31], and either affinity chromatography and hydroxyapatite chromatography [32] or ion-exchange chromatography [31], have been described. In our preparation, which includes features of both of these procedures, submitochondrial particles (200 mg protein, 30 units of transhydrogenase activity) were washed by centrifugation (200 000 × *g*, 30 min at 4°C) in 2.0 M NaCl, 10 mM Tris-HCl, 5.0 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (pH 7.8), and resuspended in 50 ml 10 mM Tris-HCl, 5 mM EDTA, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride (pH 7.8). A solution of 10% (mass/vol) Triton X-100 was added to give a final concentration of 1.5% and the mixture was incubated at 22°C for 30 min. All subsequent steps were performed at 4°C. After centrifugation (200 000 × *g*, 30 min) the supernatant was diluted 3-fold with CB medium (10 mM Tris-HCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.025% Triton X-100 (pH 7.8)) and applied to a 2.8 × 7 cm QA-Trisacryl M (IBF Biotechnics) column equilibrated with CB medium. The column was washed with CB medium until the absorbance of the eluate at 280 nm indicated that excess Triton X-100 had been displaced. H^+-Thase was eluted during application of a gradient (300 ml) of 0–1.0 M KCl in CB medium (flow rate = 1 ml min^{-1}). Fractions (6 ml) containing transhydrogenase were pooled (30 ml) and dialysed overnight against 3 L of CB medium before re-apply-

ing to a second 2.8 × 7 cm Trisacryl-QA column also equilibrated with CB medium. The column was developed with a gradient (250 ml) of 0–1.0 M KCl in CB medium (flow rate, 1 ml min^{-1}). Protein with transhydrogenase activity was eluted in a much narrower band than after the first column, partly as a result of the smaller applied volume, and perhaps also because of the lower concentration of Triton X-100. Active fractions (6 ml) were pooled (12 ml) and dialysed overnight against 2 L 5.0 mM sodium phosphate, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.025% Triton X-100 (pH 7.0) (NB medium). The protein was stored as aliquots at −80°C. The specific activity was typically in the region of 30 μmol mg^{-1} min^{-1}, and the yield approximately 6–7 units (enzyme activity during preparation was assayed in 50 mM sodium phosphate buffer (pH 6.5)). In some preparations, an additional 10 ml column of NAD^+-agarose (ribose hydroxyl-linked, Sigma type N8391), eluted with a step of KCl in NB medium, was employed between the two ion-exchange chromatography columns, but this gave only minimal improvement in the SDS-PAGE profiles. SDS-PAGE was carried out as

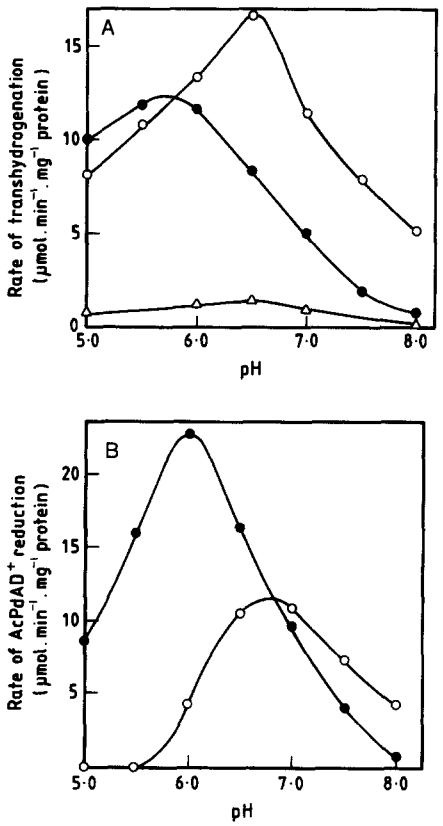

Fig. 1. pH dependences of the simple transhydrogenation reactions. (A) 10 mM Maleate, 10 mM Tricine, 0.5 mM EDTA, 0.4 mg ml^{-1} lysophosphatidylcholine and 0.025% Triton X-100. The pH was adjusted with NaOH. (B) 10 mM Mes, 10 mM Tricine, 0.5 mM EDTA, 0.4 mg ml^{-1} lysophosphatidylcholine and 0.025% Triton X-100. The pH was adjusted with NaOH. ● and ○, reduction of $AcPdAD^+$ (100 μM) by NADPH (100 μM). Δ, reduction of thio-$NADP^+$ (100 μM) by NADH (100 μM). Open symbols, no further additions; closed symbols, *plus* 10 mM $MgSO_4$.

described [23]. Protein concentrations were assayed using the bicinchoninic acid procedure, modified by inclusion of iodoacetamide to eliminate interference from dithiothreitol [37].

Transhydrogenase activity of solubilised enzyme was assayed with a Shimadzu UV3000 dual-wavelength spectrophotometer using extinction coefficients given in Ref. [29]. All assays with solubilised enzyme (10 μl) were performed with buffers (3.0 ml) described in the text *plus* 0.4 mg ml^{-1} lysophosphatidylcholine and 0.025% Triton X-100. The pH was adjusted with NaOH.

Proteoliposomes were routinely prepared by the 'cholate dilution' method [30]. Chloroform was removed under N_2 from phosphatidylcholine (Lipid Products) and the lipid resuspended to a concentration of 20 mg ml^{-1} in 10 mM sodium maleate, 10 mM Na^+-Tricine, 0.5 mM EDTA, 2 mM dithiothreitol, 5% sodium cholate (pH 6.0). The mixture was sonicated in an ice-water bath for 10 min (Branson 1210) and then diluted 5-fold in 10 mM sodium maleate, 10 mM Na^+-Tricine, 0.5 mM EDTA, 2 mM dithiothreitol (pH 6.0), and enough enzyme solution to bring the final concentration of cholate to 1% and the lipid/protein ratio to 500 (mass/mass). After 10 min incubation on ice, the suspension was diluted approximately 100-fold with 10 mM sodium maleate, 10 mM Na^+-Tricine, 0.5 mM EDTA (pH 6.0), and then incubated on ice for at least another 1 h before use. Transhydrogenase activity of the proteoliposomes was assayed directly: the suspension (3.0 ml) was transferred to the spectrophotometer cuvette and brought to 25°C before addition of the nucleotide substrates.

3. Results

3.1. Catalytic activity of transhydrogenase from beef-heart mitochondria

The procedure described above yields a preparation of H^+-Thase that is approx. 90% pure, as judged by Coomassie-stained SDS polyacrylamide gels (not shown): the apparent molecular mass of the predominant polypeptide is 110 kDa (compare the predicted 109 038 Da from the cDNA sequence [9]). The enzyme catalyses the reduction of $AcPdAD^+$ by NADPH with a specific activity of 30 μmol (mg protein)$^{-1}$ min^{-1} (80 mM sodium phosphate buffer (pH 6.3)), comparing favourably with activities described for other preparations [31,32]. As with other solubilised preparations of H^+-Thase, the rate of the 'forward' reaction (e.g., the reduction of thio-$NADP^+$ by NADH) was approx. 10-times slower than that of the reverse reaction (Fig. 1A), but, in contrast with earlier reports on this enzyme, H^+-Thase prepared as described above was stable upon storage at −80°C. Our preparation of H^+-Thase catalysed a very low rate of reduction of $AcPdAD^+$ with NADH (approx. 0.3 μmol (mg protein)$^{-1}$

min^{-1}). We attribute this activity to the presence of a low-level contaminant, since it decreased progressively during purification of NADPH → $AcPdAD^+$ transhydrogenation (not shown). Such contaminating activity was also present in detergent-solubilised membranes from *Rhodobacter capsulatus* and *Rhodospirillum rubrum* and, again, was lost during purification of NADPH → $AcPdAD^+$ transhydrogenation [23]. It will become clear (below) that the removal of contaminating protein catalysing NADH → $AcPdAD^+$ activity is an essential prerequisite for studies on cyclic transhydrogenation with enzyme-bound NADP(H).

An examination of the pH dependences of simple and cyclic transhydrogenation reactions by the *E. coli* enzyme was instrumental in the development of a model of proton translocation [24,25]. The pH dependence of the reduction of $AcPdAD^+$ by NADPH catalysed by our preparation of mitochondrial transhydrogenase is shown in Fig. 1. The dependence of the rate of reaction on the concentration of $AcPdAD^+$ and NADPH at several pH values indicated that, in the conditions of Fig. 1, the enzyme was almost saturated with nucleotide substrates. In maleate/Tricine buffer in the absence of added salt, the pH dependence was bell shaped, with a maximum rate at approximately pH 6.5 (Fig. 1A). The addition of MgSO4 (Fig. 1A) or higher concentrations of KCl or Na^+-phosphate (not shown) shifted the maximum to lower values of pH (a similar effect was shown for *E. coli* transhydrogenase [24]). Thus, at low pH, salts had a stimulatory effect, and at high pH, an inhibitory effect, on the rate of transhydrogenation. It must be stressed that, although salts had the general (presumably ionic strength) effect of shifting the pH maximum to lower values, there were, in addition, specific ion effects which distorted the pH profiles in quite different ways. These will not be described in detail, but in some instances they were quite pronounced. For example, the addition of 10 mM Na^+-Mes (pH 6.0) severely decreased the rate of reduction of $AcPdAD^+$ by NADPH by H^+-Thase in 10 mM sodium phosphate buffer (pH 6.0) (90% inhibition). The inhibitory effect of Mes was barely evident in 10 mM maleate buffer (pH 6.0), was not apparent at high pH (> 7.0), and was prevented at low pH by 10 mM $MgCl_2$ or other salts. Thus, between pH 5.5–6.0, the addition of $MgCl_2$ had a marked stimulatory effect (> 5-fold) on the rate of transhydrogenation in Mes buffer (Fig. 1B) but only a small stimulatory effect in maleate or phosphate buffer (Fig. 1A). Similar effects were seen at higher concentrations of another 'Good' buffer, Mops (50% inhibition of the reaction in maleate-Tricine (pH 6.0) buffer by 30 mM Mops, data not shown). At present we cannot account for these effects of various buffer species. It seems unlikely that the low capacity of different buffers to chelate metal ions or form complexes with nucleotides will be significant, and we therefore conclude that they arise as a result of direct interactions with the protein. We have not investigated this in detail but note that, in the membrane-bound

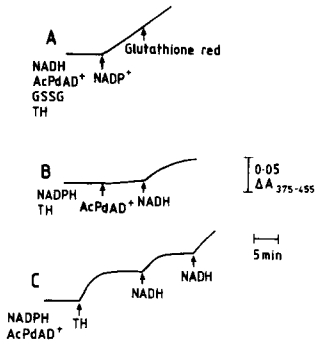

Fig. 2. NADPH-and $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH catalysed by solubilised H^+-thase. Experiments were performed in maleate/Tricine buffer (pH 6.0) (see Fig. 1A), at 30°C. The traces show the reduction of $AcPdAD^+$ at 375–455 nm. (A) The medium contained 30 μM NADH, 100 μM $AcPdAD^+$, 2 mM oxidised glutathione and 0.35 μg H^+-Thase. $NADP^+$ (100 μM) and glutathione reductase (50 μg) were then added as indicated. (B) Enzyme (0.35 μg) was preincubated with NADPH (100 μM) for 10 min, and then 100 μM $AcPdAD^+$ and 30 μM NADH were added as indicated. (C) The medium contained NADPH and $AcPdAD^+$ (100 μM each). The reaction was started by addition of H^+-thase (TH, 0.35 μg) and allowed to proceed until the rate declined to zero. Then two subsequent additions of NADH (4 μM and 30 μM) were made.

mitochondrial enzyme, in media of different ionic composition, the pH profiles and the dependence on the concentration of Mg^{2+} revealed other kinds of behaviour [33–35]. An inhibitory effect of Mes was not observed on the *E. coli* enzyme (N.P.J. Cotton and J.B. Jackson, unpublished observation). Because it seems to be relatively inert with respect to transhydrogense activity, the experiments described below were carried out in a maleate/Tricine buffer system unless otherwise stated. Although the results are not shown, $NADP^+$-and NADPH-dependent $AcPdAD^+$ reduction by NADH were observed under similar conditions to those described below, also in a phosphate buffer system.

3.2. $NADP^+$-dependent and NADPH-dependent reduction of $AcPdAD^+$ by NADH with solubilised purified transhydrogenase from mitochondria

Fig. 2A shows that, at low pH, H^+-Thase catalysed rapid reduction of $AcPdAD^+$ with a combination of $NADP^+$ and NADH. In principle, this might arise from reduction of $NADP^+$ by NADH, accumulation of NADPH in the medium (with eventual saturation of the NADPH binding site), and then subsequent reduction of $AcPdAD^+$ by NADPH. However, two observations render this explanation unlikely. (i) A similar reaction was observed when thio-$NADP^+$ replaced $NADP^+$. In this case it could be shown directly (from the absorbance change at 395 nm) that the rate of reduction of thio-$NADP^+$ by NADH in the absence of $AcPdAD^+$ was approx. 5-times less than the rate of $AcPdAD^+$ reduction by the combination of NADH and thio-$NADP^+$ (not shown). (ii) The addition of glu-

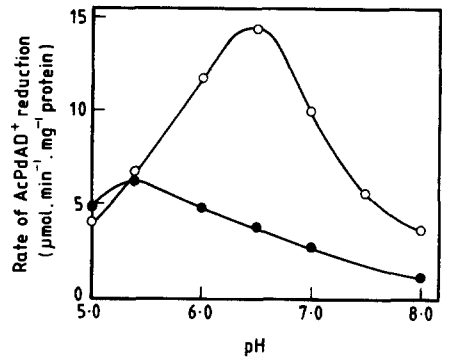

Fig. 3. pH-dependences of simple reverse transhydrogenation (○) and $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH (●) catalysed by solubilised H^+-thase. Reaction was started by addition of H^+-thase (0.25 μg) and initial rates were recorded. Maleate/Tricine medium (see Fig. 1A) contained, in addition: ○, NADPH and $AcPdAD^+$ (100 μM each); ●, 100 μM $NADP^+$, 100 μM $AcPdAD^+$, 30 μM NADH.

tathione reductase (in the presence of oxidised glutathione) during reduction of $AcPdAD^+$ by the combination of NADH and $NADP^+$ did not lead to inhibition of the reaction (Fig. 2A). In a separate experiment performed under similar conditions, it was shown that these concentrations of oxidised glutathione and glutathione reductase were sufficient to catalyse oxidation of NADPH at a rate greatly in excess of that at which the transhydrogenase could catalyse $NADP^+$ reduction. Thus, the accumulation of NADPH in the medium is not a prerequisite for $AcPdAD^+$ reduction by $NADP^+$ *plus* NADH. We propose (compare [24,25]) that $AcPdAD^+$ reduction under these conditions proceeds using NADPH which remains bound to the enzyme following the reduction of $NADP^+$ by NADH. Evidently, release of NADPH from H^+-Thase is a significant rate-limiting step during reduction of $NADP^+$ by NADH. Only low concentrations of $NADP^+$ were required to give $AcPdAD^+$ reduction with NADH (approx. 3 μM for half-maximal rates, data not shown). The pH dependence of $AcPdAD^+$ reduction by $NADP^+$ *plus* NADH is shown in Fig. 3. As with H^+-Thase from *E. coli*, the reaction is favoured by low pH but, in contrast to the bacterial enzyme [24,25], the rate of this reaction is lower than that of simple $AcPdAD^+$ reduction by NADPH at all but the lowest of pH values. Since substrate concentrations were close to saturating, this must mean that the rate of conversion of [enzyme.$NADP^+$.NADH] to [enzyme.NADPH.NAD^+] limits the rate of the cyclic reaction.

The complementary cyclic reaction, initiated with NADPH instead of $NADP^+$, was easy to demonstrate with solubilised and purified *E. coli* transhydrogenase; it was very rapid at low pH [24,25], but, as described [21,27], NADPH-dependent reduction of $AcPdAD^+$ by NADH was not observed with untreated solubilised *mitochondrial* transhydrogenase. Thus, there was no increase in the rate of $AcPdAD^+$ reduction with NADPH and NADH compared with NADPH alone. However, Fig. 2B and 2C show that this reaction did become evident after a period of incubation of the enzyme with NADPH. As briefly indicated [36], incubation of solubilised mitochondrial transhydrogenase with NADPH led to inactivation of the enzyme in its ability to perform simple $AcPdAD^+$ reduction by NADPH. Since this phenomenon has not been described in detail, a few comments are required. At 20°C and pH 6.0 in either maleate or phosphate buffer, the enzyme was 90% inactivated by 200 μM NADPH within 20 min (as evidenced by the initial rate of reduction of subsequently added $AcPdAD^+$). The NADPH was not significantly oxidised during the 20 min incubation period. A similar period of incubation in the absence of nucleotides (or in the presence of $NADP^+$, or NADH, or $AcPdAD^+$) led to only 10–15% inhibition. Low concentrations of NADPH were sufficient to promote inactivation (3–5 μM gave approximately the half-maximal rate of inhibition at pH 6.0 in 10 mM phosphate buffer). The forward transhydrogenation reaction (reduction of thio-$NADP^+$ by NADH) was similarly inhibited after incubation with NADPH. Inactivation of H^+-Thase by NADPH was very pH dependent (Fig. 4) and temperature dependent (there was no inhibition at 4°C, and the rate of inhibition at 30°C was about twice that at 20°C). Transhydrogenase, which had been inhibited by brief exposure to NADPH at low pH, was not reactivated, either with oxidised glutathione/glutathione reductase, or by increasing the pH. When transhydrogenation between $AcPdAD^+$ and NADPH was initiated (at low pH) by addition of enzyme (i.e., no preincubation with NADPH) the rate of reaction progressively decreased with time (solid line in Fig. 5). The profile of this change in rate was similar to the time-course of inactivation of the enzyme by NADPH alone (closed

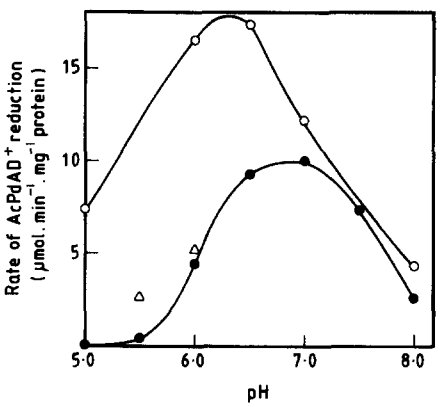

Fig. 4. Inhibition of solubilised H^+-thase by NADPH. Experiments were performed in maleate/Tricine buffer (see Fig. 1A) at 30°C. ○, the medium contained NADPH and $AcPdAD^+$ (100 μM each), the reaction was started by addition of H^+-thase (0.25 μg) and initial rates were recorded. ●, enzyme (0.25 μg) was pre-incubated with 100 μM NADPH for 8 min and reaction was then started by addition of 100 μM $AcPdAD^+$. Δ, the initial rate of reduction of $AcPdAD^+$ when, 30 s after addition of $AcPdAD^+$ in ●, NADH (30 μM) was added.

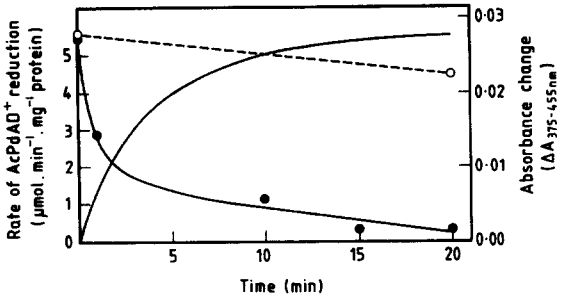

Fig. 5. Time course for the inhibition of transhydrogenase by NADPH. The reaction medium contained 10 mM sodium phosphate, 0.5 mM EDTA, 0.4 mg ml^{-1} lysophosphatidyl choline and 0.025% Triton X-100 (pH 6.0). ●, H^+-thase (0.25 μg) was incubated in the reaction medium for the time indicated in the presence of 200 μM NADPH, and then 200 μM $AcPdAD^+$ was added and the initial rate of transhydrogenation recorded. ○, similar but no NADPH was present during the preincubation period. NADPH (200 μM) was added immediately after the $AcPdAD^+$ and the initial rate of transhydrogenation recorded. The trace (solid line, absorbance scale as indicated on the right) shows the course of transhydrogenation when H^+-thase (0.25 μg) was added to the medium containing NADPH and $AcPdAD^+$ (200 μM each).

symbols in Fig. 5). This suggests that the presence of $AcPdAD^+$ does not affect the inactivation of the enzyme by NADPH.

The mechanism by which NADPH causes inactivation of bovine transhydrogenase is not clear. That NADPH binding to transhydrogenase leads to changes in the protein conformation, is well established (reviewed [1–3] and, as a minimal hypothesis, we propose that a new conformation of the enzyme becomes thermally accessible and is generated irreversibly at low pH in the presence of the nucleotide. It is suggested in the Discussion that in this conformation the pK_a of the group responsible for the proton release that accompanies the NADP(H) release is elevated. The significance of the phenomenon in the context of the present study is that NADPH-dependent $AcPdAD^+$ reduction by NADH became evident when bovine transhydrogenase was incubated with NADPH (Fig. 2). Thus, when the enzyme was partially inactivated by a short period of incubation, either with NADPH followed by $AcPdAD^+$ (Fig. 2B), or with NADPH together with $AcPdAD^+$ (Fig. 2C), then the subsequent addition of NADH led to a large increase in the rate of $AcPdAD^+$ reduction. The extent of the enhanced $AcPdAD^+$ reduction was equal to the amount of NADH supplied, and a second addition of NADH led to another period of $AcPdAD^+$ reduction (Fig. 2C), indicating that reducing equivalents for this reaction were derived entirely from the NADH. The pH dependence of NADPH-dependent reduction of $AcPdAD^+$ by NADH could not be monitored directly since the process was masked by the pH dependence of enzyme inactivation by NADPH. Thus, it is clear from Fig. 4, that the relative increase in the rate of $AcPdAD^+$ reduction upon addition of NADH was strongly influenced by the inhibition resulting from the previous period of incubation with NADPH. To investigate the pH dependence of NADPH-dependent reduction of $AcPdAD^+$ by NADH, the following strategy was adopted. Each sample was first incubated at pH 5.5 with NADPH and $AcPdAD^+$ for 15 min (to give a fixed degree of almost complete inactivation, compare Fig. 2C). The pH was then shifted to a desired value, and NADH was added immediately afterwards. In Fig. 6 the initial rate of $AcPdAD^+$ reduction upon addition of the NADH is plotted as a function of the final pH. As observed for $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH (Fig. 3), the NADPH-dependent reaction also increased with decreasing pH across the entire range.

3.3. $NADP^+$-dependent and NADPH-dependent reduction of $AcPdAD^+$ by NADH in liposomes inlaid with purified transhydrogenase from mitochondria

In preliminary experiments it was found that, in our hands, transhydrogenase proteoliposomes prepared by the cholate dilution procedure ([30] and see Methods) were better coupled than those prepared by cholate dialysis. For the former, the degree of stimulation by 1.5 μM FCCP of $AcPdAD^+$ reduction by NADPH was typically 50 fold (the rate was very low in coupled vesicles), and for the latter, 3–4 fold. Thus, proteoliposomes prepared by cholate dilution were used routinely in the following experiments.

Fig. 7A shows that the reduction of $AcPdAD^+$ by NADPH occurred at a very low rate in coupled proteoliposomes but that addition of a low concentration of NADH led to a burst of activity. As described [26], but not shown in Fig. 7, the extent of rapid $AcPdAD^+$ reduction was equal to the quantity of added NADH, and subsequent additions of NADH gave rise to further equivalent bursts. These experiments indicate that reducing equivalents for $AcPdAD^+$ reduction are derived from the NADH. The figure also shows that FCCP addition following cessation of the burst caused a large stimulation in the rate of

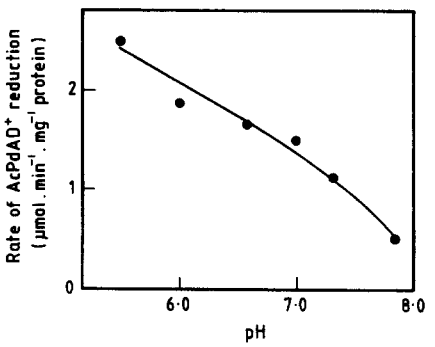

Fig. 6. pH profile of NADPH-dependent reduction of $AcPdAD^+$ by NADH catalysed by solubilised H^+-thase. Enzyme (0.35 μg) was incubated in maleate/Tricine buffer (see Fig. 1A) (pH 5.5), with NADPH and $AcPdAD^+$ (100 μM each), for 15 min at 30°C. The pH was then quickly brought to the indicated value (by addition of NaOH), 30 μM NADH was added, and the initial rate of $AcPdAD^+$ reduction was recorded.

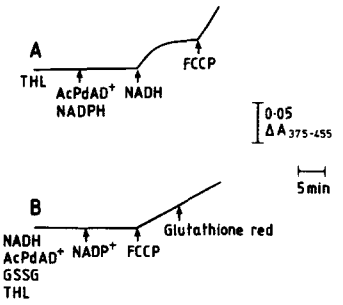

Fig. 7. NADPH-and $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH catalysed by H^+-thase reconstituted into liposomes. H^+-thase was reconstituted and assayed in maleate/tricine buffer at pH 6.0, as described in Methods. (A) To medium containing transhydrogenase liposomes (THL, 0.35 μg H^+-thase), NADPH and $AcPdAD^+$ were added (100 μM each), followed by 10 μM NADH and 1.5 μM FCCP, as indicated. (B) To medium containing liposomes, 30 μM NADH, 100 μM $AcPdAD^+$ and 2 mM oxidised glutathione were added. The trace shows the reduction of $AcPdAD^+$ upon subsequent addition of 100 μM $NADP^+$, 1.5 μM FCCP and 50 μg of glutathione reductase.

reduction of $AcPdAD^+$ by NADPH. In agreement with [21,27], addition of NADH *after* treatment with FCCP did not lead to an enhanced rate of $AcPdAD^+$ reduction (not shown). Note that $AcPdAD^+$ reduction by NADH alone in the absence (Fig. 7B) or presence (not shown) of FCCP was extremely slow.

In contrast to solubilised mitochondrial H^+-Thase (Fig. 2), the reconstituted enzyme did not require a period of exposure to NADPH before it would perform NADPH-dependent $AcPdAD^+$ reduction by NADH (Fig. 7). Nor did exposure to NADPH for up to 30 min result in inhibition of the enzyme activity ($AcPdAD^+$ reduction by NADPH). Either the detergent microenvironment of the solubilised enzyme sensitises the enzyme to NADPH inhibition, or the phospholipid microenvironment in the liposome offers protection.

In contrast to the situation with solubilised enzyme (Fig. 2A, and see above), coupled transhydrogenase vesicles were incapable of catalysing a significant rate of $AcPdAD^+$ reduction with a combination of NADH and $NADP^+$ (Fig. 7B). However, $AcPdAD^+$ reduction with NADH *plus* $NADP^+$ did ensue following the addition of uncoupler (Fig. 7B). In complementary experiments (not shown), it was established that the order of addition of the FCCP and nucleotides was not critical: $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH only took place in *uncoupled* proteoliposomes. As was found for solubilised H^+-Thase, it seems that the reaction in uncoupled proteoliposomes proceeds by way of enzyme-bound NADPH generated during the reduction of $NADP^+$ by NADH; (a) the rate of reduction of thio-$NADP^+$ by NADH with transhydrogenase proteoliposomes was approximately 5 fold less than the rate of thio-$NADP^+$-dependent reduction of $AcPdAD^+$ by NADH (not shown), and (b) the presence of oxidised glutathione and glutathione reductase at concentrations sufficient to reoxidise the NADPH in solution (i.e., generated during reduction of $NADP^+$ by NADH) did not depress the rate of $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH (Fig. 7B).

4. Discussion

The steady-state rate of $AcPdAD^+$ reduction by NADPH (simple 'reverse' transhydrogenation) catalysed by solubilised H^+-Thase from mitochondria has a complex dependence on pH and on the concentration of metal and buffer ions. This might be a reflection of the fact that, in its membrane-associated form, the enzyme functions as a proton pump. Perhaps, in detergent-solubilised transhydrogenase, the access to and the conduction along pathways for proton transfer are critically dependent on the precise local conformation of the protein, and this, in turn, is dependent on the ionic composition of the surrounding medium. However, amongst the complexities of the effects of salts and buffers on the rate of transhydrogenation by the mitochondrial enzyme there is a general response; increasing ionic strength led to a decrease in the pH optimum of $AcPdAD^+$ reduction by NADPH (Fig. 1A and 1B). *E. coli* transhydrogenase behaves similarly [24,25], and this was taken in support of other evidence that indicated a decrease in the pK_a of the group which deprotonates upon dissociation of NADP(H) from the enzyme (see below). In transhydrogenase from both sources it might suggest that protonic access to this group is decreased at elevated values of the ionic strength.

A central objective in the work described in this report was to discover whether $NADP^+$-and NADPH-dependent reduction of $AcPdAD^+$ by NADH can take place in solubilised mitochondrial H^+-Thase. In fact, the $NADP^+$-dependent reaction was observed without treatment of the enzyme, provided the medium pH was low. It was shown that $NADP^+$-dependent $AcPdAD^+$ reduction by NADH is probably a cyclic process: $NADP^+$ is first reduced by NADH (simple 'forward' transhydrogenation) and then, *while bound to the enzyme*, NADPH reduces the $AcPdAD^+$.

The complementary reaction, NADPH-dependent reduction of $AcPdAD^+$ by NADH, was not observed with untreated enzyme. Now as briefly described [36], a short period of incubation of beef-heart transhydrogenase with NADPH results in inactivation of simple 'reverse' transhydrogenation from NADPH to $AcPdAD^+$ and some of the factors influencing the inhibition process were described in Results. However, the essential observation for the purposes of this report, was that progressive inhibition of the enzyme by NADPH unmasked an NADPH-dependent reduction of $AcPdAD^+$ by NADH. As explained at length [24,25], this process consists of a set of cyclic reactions in which enzyme-bound $NADP^+$, generated during reduction of $AcPdAD^+$ by NADPH, is re-reduced by NADH. Thus, it is suggested that a critical consequence of the inhibition

of H^+-Thase by NADPH is that the rate of release of $NADP^+$ from the enzyme is considerably decreased. This results in a slowing down of simple $AcPdAD^+$ reduction by NADPH (Fig. 2, Fig. 4, Fig. 5), and an increase in the rate of NADPH-dependent $AcPdAD^+$ reduction by NADH (Fig. 2, Fig. 4, Fig. 6).

Clearly, there is no physiological significance to the fact that *E. coli* and mitochondrial H^+-Thase can perform NADP(H)-dependent reduction of $AcPdAD^+$ by NADH. However, the reactions are a useful indication that, under some conditions, the enzymes can bind $NADP^+$ and NADPH rather tightly. Particularly significant is the observation that, for the enzymes from both sources, the $NADP^+$-and the NADPH-dependent reduction of $AcPdAD^+$ *increase* in rate as the pH is progressively decreased, (Figs. 3 and 6), whereas at low pH (between 6.5 and 5.5 in mitochondria) the rates of the simple linear reactions ('forward' and 'reverse', Fig. 1) *decrease*. Since the existence of bound $NADP^+$ or NADPH favours the operation of the cyclic reaction but limit the simple transhydrogenation reactions, this suggests that the release of protons accompanies the release of both nucleotides. The situation was less complicated in the case of H^+-Thase from *E. coli*, where both the $NADP^+$-and the NADPH-dependent reduction of $AcPdAD^+$ by NADH exceeded the rate of simple $AcPdAD^+$ reduction by NADPH by approx. 10-fold [24]. We suggest that two factors account for the differences between the mitochondrial and *E. coli* enzymes. (a) The conversion of [enzyme.$NADP^+$NADH] to [enzyme .NADPH.NAD^+] is relatively slow in detergent-solubilised mitochondrial transhydrogenase and limits the maximum turnover rate of the cycle (see Results); it is relatively fast in the *E. coli* enzyme [25]. (b) The pK_a for proton release from the [enzyme.NADP(H)] complexes is less in mitochondrial transhydrogenase (approx. 5.5) than in the *E. coli* enzyme (approximately 6.5); this suggestion is consistent with the observation that the pH optimum for the simple reduction of $AcPdAD^+$ by NADPH is about one unit lower for mitochondrial enzyme (Fig. 1A) than for *E. coli* transhydrogenase [25]. It means that a lower pH is required to switch from predominantly simple forward or reverse transhydrogenation to the cyclic pathway. Thus, the $NADP^+$-dependent cycle of mitochondrial H^+-Thase is rather slow even at low pH (e.g., Fig. 3). The NADPH-dependent cycle was not detectable (at any pH) in the untreated enzyme because simple reverse transhydrogenation (from NADPH → $AcPdAD^+$), a process which, of course, includes release of $NADP^+$, is faster than the conversion of [enzyme.$NADP^+$.NADH] to [enzyme.NADPH.NAD^+] at all accessible values of pH. To explain why the cyclic reaction does become evident after a period of incubation with NADPH, it is further proposed that this treatment leads to an increase in the pK_a of [enzyme.NADP(H)]. This is supported by the shift in the pH maximum of $AcPdAD^+$ reduction by NADPH shown in Fig. 4. The increased pK_a leads, at low pH, to a decrease in the rate of $NADP^+$ release see above) to the point at which the cyclic pathway becomes favourable.

It was suggested that the protonation and deprotonation reactions associated with NADP(H) binding and release might, in the membrane-associated protein, be components of proton translocation [24,25]. Thus, during 'reverse' transhydrogenation in everted membrane vesicles, NADPH binding would be accompanied by H^+ binding at the outside, and $NADP^+$ release would be accompanied by H^+ release at the inside of the membrane surface. A mechanism was proposed in which the value of the pK_a of a group, X, located in the proton translocation channel, increases upon NADP(H) binding. When NADPH is bound to the protein, X is only accessible to protons in the external aqueous phase and, when $NADP^+$ is bound, X is only accessible to protons in the internal aqueous phase. Accessibility is controlled by barriers to proton conduction whose operation is conformationally synchronised with changes in the pK_a of X. In support of the view that $NADP^+$ release is accompanied by H^+ release on the inside of a vesicle we cited [24] the observation that NADPH-dependent reduction of $AcPdAD^+$ by NADH in proteoliposomes was abolished by uncouplers (Refs. [21,27] and Fig. 7A, above): the elevated value of the internal proton electrochemical potential in coupled membranes (resulting from inward H^+ translocation) would limit internal H^+ release and therefore keep the $NADP^+$ bound to the protein. After addition of uncoupler, the fall in the internal proton potential would favour release of the nucleotide. Note that the effect observed in coupled membranes can be achieved in the solubilised enzyme (after incubation with NADPH) simply by lowering the pH (see above).

Offering further support to the proposed mechanism of proton translocation, is the finding (Fig. 7B) that, in contrast, $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH in proteoliposomes is only observed in the ***presence*** of uncoupler. With $NADP^+$, NADH and $AcPdAD^+$ as nucleotide substrates, the internal proton electrochemical potential will be relatively low in coupled vesicles due to the outward proton translocation which accompanies the reduction of $NADP^+$ by NADH (note the uncoupler stimulation of thio-$NADP^+$ reduction by NADH, described in Results). Consistent with the hypothesis, the low internal proton potential (equivalent to a high pH value) would favour the release of $NADP^+$ and limit the rate of re-binding of $NADP^+$ to the enzyme. Thus, the cyclic reaction would not occur. After dissipation of the proton electrochemical gradient with uncoupler, $NADP^+$ binding would become more favourable. Since, after addition of uncoupler, the pH inside the vesicle would be close to 6.0 (the pH of the external medium), this is low enough to ensure that the release of NADPH from the enzyme is still rather slow and therefore that the cycle will proceed at a substantial rate.

In conclusion, we have shown that cyclic, NADP(H)-dependent reduction of $AcPdAD^+$ by NADH [21,24–27] *does* occur in some circumstances in solubilised mitochondrial transhydrogenase, despite assertions to the contrary [21,27]. Taken together with observations on the reaction in reconstituted liposomes (above and Refs. [21,27]), this provides strong support for the hypothesis advanced [24,25] that NADP(H) binding/release is accompanied by the binding/release of translocated protons.

Acknowledgements

L.A.S. is grateful to the Wellcome Trust for a Research Fellowship. Support from the Biotechnology and Biological Sciences Research Council is also acknowledged. We thank our colleagues. Tania Bizouarn, Mike Hutton and Nick Cotton, for advice and valuable discussion.

References

[1] Jackson, J.B. (1991) J. Bioenerg. Biomembr. 23, 715–741.
[2] Hatefi, Y. and Yamaguchi, M. (1992) in Molecular mechanisms in bioenergetics (Ernster, L., ed.), pp. 265–281, Elsevier, Amsterdam.
[3] Rydstrom, J., Persson, B. and Carlenor, E. (1987) in Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects, Vol. 2B (Dolphin, D., Poulson, R. and Avramovic, O., eds.), pp. 433–460, John Wiley and Sons, New York.
[4] Sazanov, L.A. and Jackson, J.B. (1994) FEBS Lett. 344, 109–116.
[5] Hanson, R.L. and Rose, C. (1980) J. Bacteriol. 141, 401–404.
[6] Clarke, D.M., Loo, T.W., Gillam, S. and Bragg, P.D. (1986) Eur. J. Biochem. 158, 647–653.
[7] Yamaguchi, M., Hatefi, Y., Trach, K. and Hoch, J.A. (1988) J. Biol. Chem. 263, 2761–767.
[8] Ahmad, S., Glavas, N.A. and Bragg, P.D. (1992) Eur. J. Biochem. 207, 733–739.
[9] Holmberg, E., Olausson, T., Hultman, T., Rydstrom, J., Ahmad, S., Glavas, N.A. and Bragg, P.D. (1994) Biochemistry 33, 7691–7700.
[10] Kramer, R.A., Tomchak, L.A., McAndrew, S.J., Becker, K., Hug, D., Pasamontes, L. and Humbelin, M. (1993) Mol. Biochem. Parasitol. 60, 327–332.
[11] Williams, R., Cotton, N.P.J., Thomas, C.M. and Jackson, J.B. (1994) Microbiology 140, 1595–1604.
[12] Yamaguchi, M. and Hatefi, Y. (1994) J. Bioenerg. Biomembr. 26, 435–445.
[13] Yamaguchi, M., Wakabayashi, S. and Hatefi, Y. (1990) Biochemistry 29, 4136–4143.
[14] Yamaguchi, M. and Hatefi, Y. (1991) J. Biol. Chem. 266, 5728–5735.
[15] Cunningham, I.J., Williams, R., Palmer, T., Thomas, C.M. and Jackson, J.B. (1992) Biochim. Biophys. Acta 1100, 332–338.
[16] Tong, R.C.W., Glavas, N.A. and Bragg, P.D. (1991) Biochim. Biophys. Acta 1080, 19–28.
[17] Wakabayashi, S. and Hatefi, Y. (1987) Biochem. Internat. 15, 915–924.
[18] Yamaguchi, M. and Hatefi, Y. (1993) J. Biol. Chem. 268, 17871–17877.
[19] Hu, P.S., Persson, B., Hoog, J.O., Jornvall, H., Hartog, A.F., Berden, J.A., Holmberg, E. and Rydstrom, J. (1992) Biochim. Biophys. Acta 1102, 19–29.
[20] Hanson, R.L. (1979) J. Biol. Chem. 254, 888–893.
[21] Enander, K. and Rydstrom, J. (1982) J. Biol. Chem. 257, 14760–14766.
[22] Homyk, M. and Bragg, P.D. (1979) Biochim. Biophys. Acta 571, 201–217.
[23] Lever, T.M., Palmer, T., Cunningham, I.J., Cotton, N.P.J. and Jackson, J.B. (1991) Eur. J. Biochem. 197, 247–255.
[24] Hutton, M.N., Day, J.M. and Jackson, J.B. (1994) Eur. J. Biochem. 219, 1041–1051.
[25] Bizouarn, T., Grimley, R.L., Cotton, N.P.J., Stilwell, S.N., Hutton, M. and Jackson, J.B. (1995) Biochim. Biophys. Acta 1229, 49–58.
[26] Wu, L.N.Y., Earle, S.R. and Fisher, R.R. (1981) J. Biol. Chem. 256, 7401–7408.
[27] Fisher, R.R. and Earle, S.R. (1982) in The Pyridine Nucleotide Coenzymes (Everse, J., Anderson, B.M. and You, K.S., eds.), pp. 279–324, Academic Press, New York.
[28] Low, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–374.
[29] Palmer, T. and Jackson, J.B. (1992) Biochim. Biophys. Acta 1099, 157–162.
[30] Eytan, G.D., Persson, B., Ekebacke, A. and Rydstrom, J. (1987) J. Biol. Chem. 262, 5008–5014.
[31] Persson, B., Enander, K., Tang, H.L. and Rydstrom, J. (1984) J. Biol. Chem. 259, 8626–8632.
[32] Phelps, D.C. and Hatefi, Y. (1984) Biochemistry 23, 4475–4480.
[33] Galante, Y.M., Lee, Y. and Hatefi, Y. (1980) J. Biol. Chem. 255, 9641–9646.
[34] O'Neal, S.G., Earle, S.R. and Fisher, R.R. (1980) Biochim. Biophys. Acta 589, 217–230.
[35] Sazanov, L.A. and Jackson, J.B. Jackson (1993) Biochim. Biophys. Acta 1144, 225–228.
[36] Pennington, R.M. and Fisher, R.R. (1981) J. Biol. Chem. 256, 8963–8969.
[37] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.