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The pathway of electron transfer in NADH:Q oxidoreductase

R. van Belzen and S.P.J. Albracht

Laboratory of Biochemistry, University of Amsterdam, Amsterdam (The Netherlands)

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The pre-steady-state reduction by NADPH of NADH:Q oxidoreductase, as present in submitochondrial particles, has been further investigated with the rapid-mixing, rapid-freezing technique. It was found that trypsin treatment, that had previously been used to inactivate the transhydrogenase activity (Bakker, P.T.A. and Albracht, S.P.J. (1986) Biochim. Biophys. Acta 850, 413–422), considerably affected the stability at pH 6.2 of the NAD(P)H oxidation activity of submitochondrial particles. Use of the inhibitor butadione circumvented this problem, thus allowing a more careful investigation of the kinetics at pH 6.2. In the presence of the inhibitor rotenone it was found that 50% of the Fe-S clusters 3 and all of the Fe-S clusters 2 and 4 could be reduced by NADPH within 30 ms at pH 6.2. The remainder of the Fe-S clusters 3 and all of the Fe-S clusters 1 were reduced slowly (complete reduction only after more than 60 s). It was concluded that these latter Fe-S clusters play no role in the NADPH oxidation activity. In the absence of rotenone at pH 6.2 only 50% of the Fe-S clusters 2–4 could be reduced within 30 ms, while Fe-S cluster 1 was again not reduced. This difference was attributed to the fast reoxidation of part of the Fe-S clusters 2 and 4 by ubiquinone. At pH 8.0, where the NADPH oxidation activity is almost zero, 50% of the Fe-S clusters 2–4 could still be reduced by NADPH within 30 ms, while Fe-S cluster 1 was not reduced. The presence of rotenone had no effect on this reduction. From these observations it is concluded that the Fe-S clusters 2 and 4, which were rapidly reduced by NADPH and reoxidised by ubiquinone at pH 6.2, could not be reduced by NADPH at pH 8.0. This provides an explanation why NADH:Q oxidoreductase was not able to oxidise NADPH at pH 8.0, while part of the Fe-S clusters were still rapidly reduced. As a working hypothesis a dimeric structure for NADH:Q oxidoreductase is proposed. One protomer (B) contains FMN and Fe-S clusters 1–4 in equal amounts; the other protomer (A) is identical except for the absence of Fe-S cluster 1. NADH is able to react with both protomers, while NADPH only reacts with protomer A. A pH-dependent electron transfer from protomer A to protomer B is proposed, which would allow the reduction of Fe-S clusters 2 and 4 of protomer B by NADPH at pH 6.2, which is required for NADPH:Q oxidoreductase activity.

Introduction

NADH:Q oxidoreductase (EC 1.6.99.3) is as yet the least understood enzyme complex of the mitochondrial respiratory chain. The best characterised preparation is purified from bovine mitochondria and is often called Complex I. It consists of 26 polypeptides [1–3] and contains FMN, Fe and acid-labile S, as well as phospholipids (0.22 g per g protein [4]). On the basis of flavin contents the molecular weight is estimated to be 700000 per FMN molecule [4]. At least 16 Fe and 16 acid-labile sulphide atoms per FMN have been found in the purest preparations [4] and are contained in Fe-S clusters [5]. From EPR studies with ^{57}Fe-enriched submitochondrial particles from yeast the presence of a $[2Fe\text{-}2S]^{2+(2+,1+)}$ (cluster 1) and three different $[4Fe\text{-}4S]^{2+(2+,1+)}$ clusters (clusters 2–4) was proposed a decade ago [6]. Although this proposal has been questioned at one time [7], results of recent magnetic circular dichroism (MCD) studies [8] were in line with the proposal. A fifth cluster, called cluster 1a and proposed to be a [2Fe-2S] cluster, has been reported by the group of Ohnishi [9–11]. It distinguishes itself from the other clusters mentioned above in that it cannot be reduced by NADH, whereas the other clusters are fully reduced by this substrate. The redox potential of this cluster was reported to be −385 mV in intact systems [12] to −500

Abbreviations: $AcPyAD^+$, oxidised 3-acetylpyridine adenine dinucleotide; AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; Mes, 4-morpholineethanesulphonic acid; MCD, magnetic circular dichroism;

Correspondence: S.P.J. Albracht, Laboratory of Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands.

mV in purified Complex I [13]. Other research groups could not find this cluster [14].

Quantifications based on EPR or MCD spectra all indicated the presence of equivalent amounts of the clusters 2–4, which are all present in the same concentration as FMN. There is no agreement on the amount of cluster 1. Whereas several groups reported cluster 1 concentrations equal to those of cluster 2, results obtained in this laboratory, using computer simulations as the basis for the double integrations of the experimental spectra, consistently pointed to the presence of only one cluster 1 per two clusters 2 in submitochondrial particles [15,16]. The reason for this discrepancy is as yet unclear.

A sixth cluster was observed in bovine heart submitochondrial particles as well as in Complex I [10,17,18], but its concentration is only 6–25% of that of FMN [12,15,19], while it is not present in yeast [15] or plant mitochondria [20]. For an extensive review on the properties of these Fe-S clusters the reader is referred to Ref. 14.

Another well-characterised enzyme preparation that can oxidise NADH, is the one solubilised by treating submitochondrial particles with phospholipase obtained from snake venom [3]. This enzyme, which contains very little phospholipid (0.062 g per g protein [21]), has lost the ability to react with Q_{10} [22,23]. Otherwise its FMN, iron and acid-labile sulphide contents are very similar to those of Complex I [3]. This enzyme is usually referred to as soluble, high-molecular-weight (type 1) NADH dehydrogenase.

Little is known about the path of reducing equivalents donated by NADH on their way to Q_{10}. Since NADH delivers two reducing equivalents at a time, it is generally agreed upon that FMN is the primary acceptor. Thereafter, reducing equivalents have to be distributed one at a time, since Fe-S clusters function as one-electron redox groups. It has been observed a long time ago [5] that full reduction of all four Fe-S clusters takes place within 5 ms after mixing enzyme with NADH, even at 4°C, so from this reaction no insight into the pathway of electrons could be gained. Fortunately, besides NADH oxidation, the enzyme is also capable of NADPH oxidation. Submitochondrial particles show a rotenone-sensitive NADPH oxidase activity with a rather sharp optimum around pH 6.0, which differs from the broad optimum of the NADH oxidase activity around pH 7 [24]. The K_m for NADPH in this reaction has been determined to be 550 μM, two orders of magnitude higher than the one for NADH, whereas the V_{max} of the NADPH oxidase, measured at its optimal pH, is about 60–70% of the V_{max} of the NADH oxidase at the same pH [24]. In 1965 Beinert et al. [25] showed that NADPH is able to reduce cluster 1 completely in soluble, high-molecular-weight (type 1) NADH dehydrogenase under anaerobic conditions, with a half-time of about 20 s. Later, Hatefi and Hanstein [24] and Hatefi and Bearden [26] showed that aerobic reduction of Complex I with NADPH in the presence of rotenone resulted in incomplete reduction of clusters 1 and 3. The g_z peak of cluster 3 was shifted upfield compared to its position after reduction by NADH. Also reduction of the flavin by NADPH, measured as the bleaching at 475 nm, was incomplete in Complex I as well as in rotenone-blocked, trypsin-treated submitochondrial particles [24,27]. Trypsin treatment at 0°C inactivates the energy-linked NADH-$NADP^+$ transhydrogenase activity; the NADPH oxidation activity is hardly affected by such a treatment. Because of incomplete reduction by NADPH of the flavin and Fe-S clusters 1 and 3 in Complex I, but complete reduction of the Fe-S clusters 2 and 4, Hatefi and Hanstein [24] and Hatefi et al. [28] suggested that NADPH possibly delivered reducing equivalents to the respiratory chain at the level of clusters 4 and 2, and by-passed the flavin and clusters 1 and 3. They also discovered that more severe treatment of submitochondrial particles with trypsin at 30°C specifically inactivated the NADPH oxidation activity [27,28] and they suggested independent routes for input of electrons from NADH and NADPH.

In 1976, Hatefi and Bearden [26] examined the differences in the reduction of Complex I by NADH, AcPyADH, NADPH and NADPH plus NAD^+ in more detail and concluded that these differences were essentially quantitative in nature, mainly reflecting the balance between the dehydrogenation rates of these nucleotides by Complex I and electron leakage from the complex by auto-oxidation. Simultaneously, Ragan [29,30] drew the same conclusion from the observation of the almost complete reduction of the chromophores bleaching at 450 nm, upon reduction with NADPH under anaerobic conditions. Thus, the conclusion was drawn that NADH and NADPH were oxidised at the same site, but with different rates and different pH dependencies. The differences in pH optima for the oxidation of the two substrates was explained by Galante and Hatefi [31] as being caused by the required protonation of the 2′-phosphate group of NADPH, with its pK_a of 6.1, which at low pH makes NADPH a closer electronic analogue of NADH. One of us has questioned these conclusions some time ago [32], using the argument that the rate of oxygen uptake by submitochondrial particles in the presence of NADPH at pH 6.0 is at least for 98% sensitive to rotenone. Thus the rate of auto-oxidation of Complex I is at most 2% of the dehydrogenation rate of NADPH and a higher level of reduction of cluster 1 (and of cluster 3) by NADPH might therefore be expected. It was suggested (as Hatefi and Hanstein had also initially concluded [24]) that part of these clusters might not be involved in NADPH oxidation. This was confirmed by the results from rapid-mixing rapid-freezing experiments [33,34]. Cluster

1 and part of cluster 3 seemed not to be involved in pre-steady-state kinetics of NADPH oxidation by Complex I in trypsin-treated submitochondrial particles, confirming the early proposal [32] of two independent pathways of electron transfer through NADH:Q oxidoreductase. In the present report the reaction of submitochondrial particles with NADPH has been investigated in further detail, using the working hypothesis of Albracht and Bakker [34] as a starting point.

Materials and Methods

NADPH, NADH, NAD^+ and $NADP^+$ were purchased in the purest form available from Boehringer (Mannheim). Butadione was obtained from E. Merck (Darmstadt), and $AcPyAD^+$ and AcPyADH from Sigma Chemical Co Deisenhofen (F.R.G.). All other chemicals were of analytical grade.

Bovine heart submitochondrial particles were prepared essentially as described in Ref. 35. Unless otherwise stated, the particles were treated with butadione as described in Ref. 27, except that incubation was performed at pH 8.0 and 4°C for 48 h after which the particles were washed with 0.25 M sucrose/5 mM borate/1 mM butadione (pH 8.0), and stored in liquid nitrogen. The transhydrogenase activity [36] could only be inhibited for 99% or more when butadione was kept present in the buffers. Before and after butadione treatment, the NAD(P)H oxidation activity, the NAD(P)H-ferricyanide activity [37] and the NADPH-$AcPyAD^+$ transhydrogenase activity [36] were measured. Assays for the oxidation activities of NADH and NADPH were carried out polarographically at 22°C, using a Clark electrode for measuring oxygen consumption, in the media: 0.25 M sucrose/100 mM Tris-HCl/1 mM butadione (pH 8.0) or 0.25 sucrose/100 mM Mes-KOH/1 mM butadione (pH 6.2). The cell volume was 1.60 ml and about 0.5 mg of protein of submitochondrial particles was added together with 5 mM NADH or NADPH. The noncatalytic formation of NADH was measured by mixing 5 mM NADPH with 2 mM NAD^+ in 0.25 M sucrose/100 mM Tris-HCl/1 mM butadione (pH 8.0) at 22°C. The extent of the rapid O_2-uptake in the standard NADH oxidase assay was taken as a measure for the amount of NADH formed. Q_{10} extraction was performed on submitochondrial particles, after extensive washing with a 150 mM KCl solution and lyophilization, with three washes of pentane followed by two washes with pentane that contained 10% of acetone as has been described [38]. Re-incorporation was performed with one-tenth of the extracted particles and the combined pentane extracts as has been described [38]. The amount of ubiquinone in particles was measured chemically [39].

Rapid-mixing rapid-freezing experiments were performed at room temperature (22°C) as described in Ref. 40. Two types of experiment were carried out. The first type made use of one mixing chamber and two syringes of equal size as has been described in Ref. 40 and will be called a two-syringe experiment. The other type of experiment made use of two mixing chambers and three syringes of equal size as has been described by Bray [41] and will be called a three-syringe experiment. For long reaction times (more than 10 s) the reaction time after the first mixing chamber was determined by the 'push-push' method described by Bray [41]. For shorter times the length of the tubing connecting the first mixing chamber with the second mixing chamber determined this reaction time in the normal mode of operation (single push). Strict anaerobic conditions during rapid-mixing rapid-freezing experiments were obtained by carrying out the experiment in a glove box purged with argon.

EPR spectroscopy and determination of spin concentrations were performed as has been described in Ref. 42. The amplitudes of the signals from the Fe-S clusters were determined relative to those obtained after full reduction by NADH [33]: for cluster 1 ($g_{x,y,z}$ = 1.92, 1.94, 2.02) the amplitude of the g = 1.94 line was used (50 K); for cluster 2 ($g_{x,y,z}$ = 1.92, 1.92, 2.05) the amplitude of the g = 1.92 line was used (14 K); for cluster 3 ($g_{x,y,z}$ = 1.88, 1.94, 2.10) the amplitude of the g = 1.88 line was used (8 K) and for cluster 4 ($g_{x,y,z}$ = 1.86, 1.93, 2.04) the amplitude of the g = 1.86 line was used (8 K). We have not been able to observe an additional cluster 1 (called cluster N-1a) reported by Ohnishi et al. [13], who referred to the NADH-reducible cluster 1 as N-1b (see also Ref. 14). No changes in the lineshapes of the EPR spectra of the various Fe-S clusters could be detected after incubation of submitochondrial particles with butadione, 1% (v/v) ethanol or rotenone. Rigorous extraction of Q_{10} resulted in slight broadening of the signal of Fe-S cluster 2 (9% increase of linewidth of the g = 1.92 line under standard [33] conditions).

Results

Inhibition of NADPH-$AcPyAD^+$ transhydrogenase

When repeating the original experiments of Bakker and Albracht [33,34] it was noted that after trypsin treatment submitochondrial particles were highly unstable at pH 6.2: NAD(P)H oxidation activities diminished by 50% within 30 min at 30°C (Fig. 1). Furthermore, trypsin treatment could only destroy 95% of all NADPH-$AcPyAD^+$ transhydrogenase activity at pH 6.2; a NADPH-$AcPyAD^+$ transhydrogenase activity of 20–30 nmol per min per mg protein of submitochondrial particles remained. Longer incubation of submitochondrial particles with trypsin, to destroy this residual NADPH-$AcPyAD^+$ transhydrogenase activity, seriously affected the NAD(P)H oxidation activities. Butadione, another well-known inhibitor of transhydro-

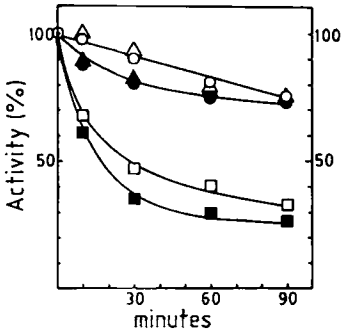

Fig. 1. The stability of NAD(P)H oxidation activities of submitochondrial particles during incubation at pH 6.2 and 30° C. Submitochondrial particles were incubated at a concentration of 40 mg protein per ml in 0.25 M sucrose/100 mM Mes-KOH (pH 6.2), while in the case of butadione-treated particles 1 mM of butadione was added to the buffer. Assays were performed at pH 6.2 as described in Materials and Methods. The activities were compared to the original activities of the untreated submitochondrial particles. ○———○, NADH oxidation of untreated submitochondrial particles; ●———●, NADPH oxidation of untreated submitochondrial particles; △———△, NADH oxidation of butadione-treated submitochondrial particles; ▲———▲, NADPH oxidation of butadione-treated submitochondrial particles,; □———□, NADH oxidation of trypsin-treated submitochondrial particles; ■———■, NADPH oxidation of trypsin-treated submitochondrial particles.

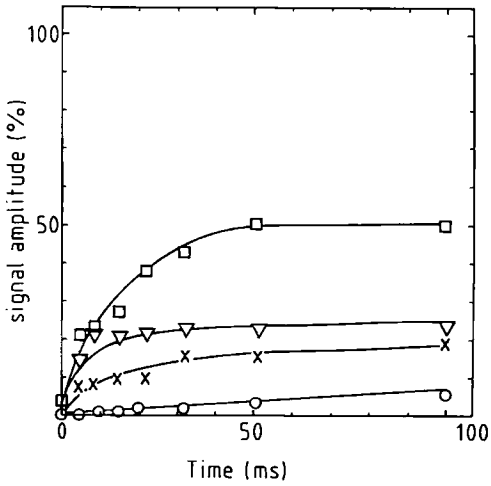

Fig. 2. Kinetics of reduction by NADPH at pH 8.0 and under anaerobic conditions of the four Fe-S clusters in NADH:Q oxidoreductase present in submitochondrial particles. Butadione-treated submitochondrial particles in 0.25 M sucrose/100 mM Tris-HCl/1 mM butadione (pH 8.0) were mixed with 10 mM NADPH dissolved in the same medium. Circles, cluster 1; squares, cluster 2; crosses, cluster 3; triangles, cluster 4.

genase activity, was able to inhibit this activity almost completely without affecting the NAD(P)H oxidation activities of submitochondrial particles and their stability at pH 6.2 (Fig. 1). The range of absolute activities of several batches of butadione-treated submitochondrial particles are shown in Table I. As can be seen, butadione treatment affected the NADH oxidation activity for less than 10%, the NADPH oxidation activity for about 5%, but the NADPH-AcPyAD$^+$ transhydrogenase activity for at least 99% (pH 6.2). We determined that the concentration of Fe-S cluster 2 in these submitochondrial particles was 0.13 nmol/mg of protein; with that information it can be calculated from Table I that the inverse turnover number of NADPH oxidation at 22° C was 30–34 ms at pH 6.2.

TABLE I

Effect of butadione on several activities of bovine-heart submitochondrial particles

All activities are expressed in nmol per min per mg protein of submitochondrial particles and depict the range of several different batches. The activities were measured at 22° C as described in Materials and Methods.

	Untreated		Butadione-treated	
	pH 6.2	pH 8.0	pH 6.2	pH 8.0
NADH oxidation	700–850	1350–1600	600–750	1200–1500
NADPH oxidation	235–275	4– 8	220–250	4– 7
NADPH-AcPyAD$^+$ transhydrogenase	560–620	70– 90	2– 4	0

Reaction with NADPH at pH 8.0

Two-syringe experiments at pH 8.0 in which NADPH was mixed with butadione-treated submitochondrial particles under strictly anaerobic conditions gave fast reduction of 40–50% of the Fe-S clusters 2 and part of clusters 3 and 4 (Fig. 2) and these levels of reduction, reached within 30 ms, remained constant up to 800 ms (not shown). The level of reduction of Fe-S cluster 1 remained below 10%. The same experiment, performed in the presence of the inhibitor rotenone, gave the same result (data not shown). When both experiments were repeated under aerobic conditions the same results were obtained (data not shown). Also the addition of 30 μM of the uncoupler 5-chloro-3-tert-butyl-2′-chloro-4′-nitro-salicylanilide (S13) to the particles gave the same results (data not shown).

Effect of NAD$^+$

When the two-syringe experiments mentioned above were repeated in the presence of additional NAD$^+$, some remarkable effects could be observed as has already been reported by Bakker and Albracht [33]. However, in contrast to these previous results, the presence or absence of rotenone strongly influenced these effects. In both cases all Fe-S clusters reached a higher level of reduction within 30 ms. In the absence of rotenone these levels dropped again after 50 ms (Fig. 3A) to a final level of reduction equal to the reduction in the absence of NAD$^+$ and remained at that level up to 800 ms (not shown). When rotenone was present, clusters 2 and 4 were fully reduced within 30 ms and clusters 1 and 3 were about half reduced (Fig. 3B). These effects were not observed when NADP$^+$ instead of NAD$^+$ was

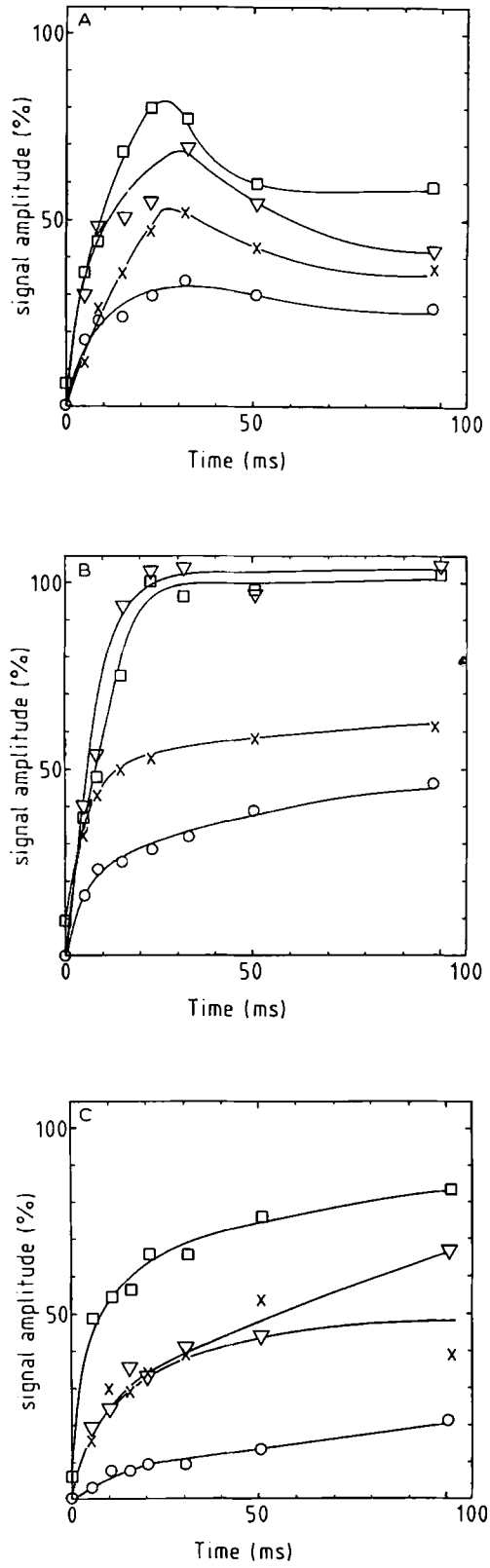

Fig. 3. Kinetics of the reduction by NADPH at pH 8.0, and in the presence of additional NAD^+, of the four Fe-S clusters in NADH:Q oxidoreductase present in submitochondrial particles. (A) Conditions were as in Fig. 2, except for aerobicity and the addition of 4 mM NAD^+ to the NADPH solution. (B) 30 μM rotenone was added to the submitochondrial particles. (C) As (B), except that the 4 mM NAD^+ was added to the submitochondrial particles instead of to the NADPH prior to mixing. The symbols used are the same as in Fig. 2.

added (data not shown). The addition of 2 mM $NADP^+$ together with 5 mM NADPH even lowered the levels of reduction for all Fe-S clusters, as compared to the results in Fig. 2. Since a small amount of NADH, formed by non-catalytic transfer of reducing equivalents from NADPH to NAD^+ prior to the mixing of the submitochondrial particles with substrate, might explain all these effects, the substrate mixture was tested for the presence of NADH by its ability to consume oxygen in the presence of submitochondrial particles at pH 8.0. The formation of small amounts of NADH was indeed established. The rate of NADH formation proved to be very small. Under our experimental conditions it was 1–2 μM/min. This, however, was enough to explain all these effects of NAD^+ that were observed in Fig. 3A and B. For this reason the experiment was repeated by adding NAD^+ to the submitochondrial particles prior to mixing, instead of to the NADPH solution (Fig. 3C). As compared to Fig. 2B, half of the clusters 2 reduced much faster in the presence of NAD^+. Additional slow reduction of the clusters 2 (and also of the clusters 4) was observed with an apparent rate of 15 nmol per min per mg protein. Non-catalytic formation of NADH could only contribute to this rate for 1%, whereas the NADPH-AcPyAD$^+$ transhydrogenase activity was completely inhibited at pH 8.0. With the same batch of particles the oxidation of NADPH at pH 8.0 was 4 nmol per min mg per protein, while addition of 2 mM NAD^+ resulted in a rotenone-sensitive oxidation rate of 12 nmol min per mg protein.

Reaction with NADPH at pH 6.2

The experiments shown in Figs. 2 and 3 were repeated at pH 6.2. In the absence of rotenone and NAD^+, and under anaerobic conditions, clusters 2 and 4 reached 50% reduction within 30 ms, while the clusters 3 were 30% reduced and the clusters 1 only 10% (Fig. 4A). When rotenone was present, the reduction of clusters 1 remained low, while the clusters 3 reached 30% reduction and clusters 2 and 4 were almost 90% reduced within 50 ms (Fig. 4B). The reduction of the clusters 3 eventually reached 50% and remained 50% up to 800 ms, while clusters 2 and 4 were fully reduced at $t = 200$ ms (data not shown). Cluster 1 reduction remained below 25% within this same range. These experiments were repeated under aerobic conditions and gave the same results. At this pH, the addition of NAD^+ had no effect. Also the addition of 30 μM of the uncoupler 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide (S13) to the particles gave the same results (data not shown).

Reaction of Q_{10}-free submitochondrial particles with NADPH

Since some investigators had suggested a role for ubiquinone in the functioning of Complex I, which

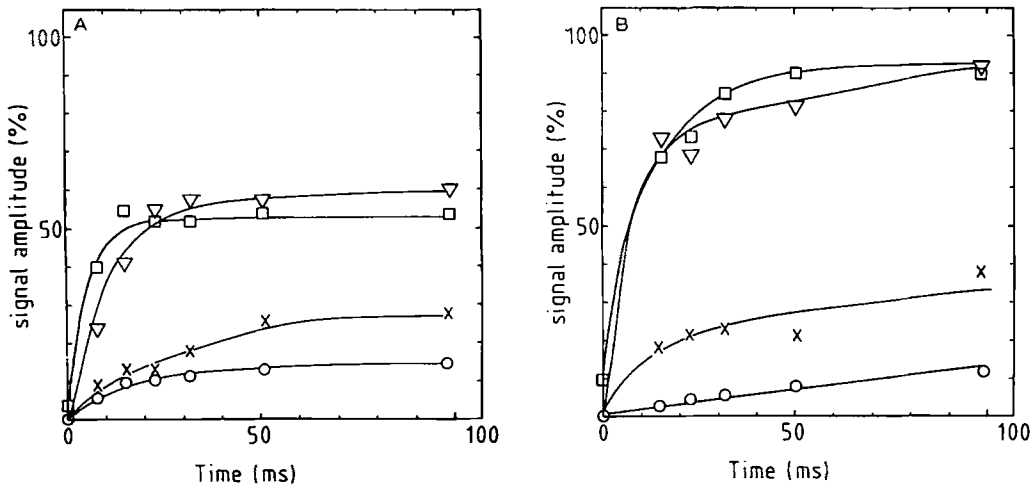

Fig. 4. Kinetics of the reduction by NADPH at pH 6.2 and under anaerobic conditions of the four Fe-S clusters in NADH:Q oxidoreductase present in submitochondrial particles. (A) Butadione-treated submitochondrial particles in 0.25 M sucrose/100 mM Mes-KOH/1 mM butadione (pH 6.2) were mixed with 10 mM NADPH dissolved in the same medium. (B) As (A), except for the addition of 30 μM rotenone to the submitochondrial particles. The symbols are the same as in Fig. 2.

would be reflected in the route of electron transfers within the enzyme [43], it was decided to do rapid-mixing rapid-freezing experiments with Q_{10}-free submitochondrial particles. These particles had lost their ability to oxidise both NADH and succinate and these activities could only be partially restored by re-incorporation of ubiquinone (35% of the NADH oxidation activity and 65% of the succinate oxidation activity) as was reported [38]. NADH-ferricyanide activity was not destroyed but altered, as shown in Fig. 5. This alteration was not restored by re-incorporation of ubiquinone

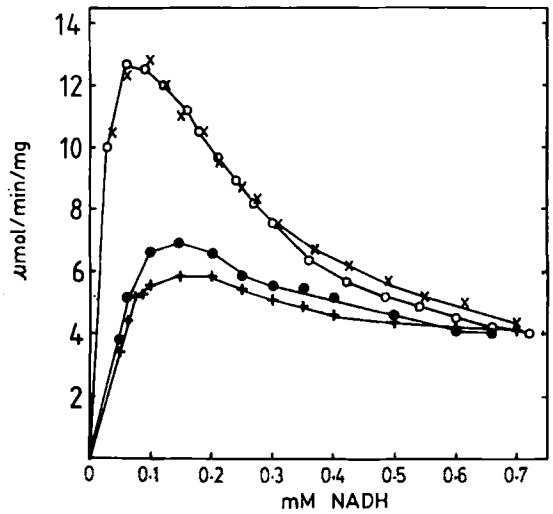

Fig. 5. The NADH-ferricyanide activities of bovine heart submitochondrial particles as a function of NADH concentration at pH 8.0 and 22° C. The ferricyanide concentration was 1 mM in 0.25 M sucrose/100 mM Tris-HCl (pH 8.0). The activity is expressed as μmol ferricyanide reduced per min per mg protein of submitochondrial particles. o——o, untreated, ×——×, butadione-treated (1 mM of butadione was added to the buffer); +——+, Q_{10}-free; ●——●, Q_{10}-free submitochondrial particles after re-incorporation of Q_{10}.

(Fig. 5). The EPR spectra of the Fe-S clusters of Complex I in submitochondrial particles did not change upon removal of Q_{10}, except for a slight broadening (9%) of the $g = 1.92$ line of Fe-S cluster 2 recorded at 17 K and 0.2 mW. At lower temperatures the signal sharpened up, so the broadening at 17 K was probably due to an increase in spin-lattice relaxation. Re-incorporation of Q_{10} did not restore the original relaxation behaviour of this Fe-S cluster. In Q_{10}-free submitochondrial particles complete reduction of all Fe-S clusters was obtained within 5 ms, both at pH 8.0 and at pH 6.2, when mixed with NADH (5 mM). When butadione-treated, Q_{10}-free submitochondrial particles were mixed with NADPH, the reduction of the Fe-S clusters of Complex I at pH 8.0 was virtually identical to the reduction in normal submitochondrial particles (Fig. 6). When mixed with NADPH at pH 6.2, the reduction of the Fe-S clusters (Fig. 6B) strongly resembled the reduction of untreated particles in the presence of rotenone (Fig. 4B). However, reduction was more complete: clusters 2 and 4 were fully reduced within 30 ms, whereas clusters 3 were half reduced by then. Reduction of clusters 1 seemed higher than in normal submitochondrial particles, due to a contribution of an unknown overlapping signal. The appearance of this extra signal was not observed in normal submitochondrial particles; its shape resembled the one obtained with mitochondrial outer membranes [45]. The experiments of Fig. 6 were repeated in the presence of rotenone with identical results (data not shown).

Reoxidation experiments

In the working hypothesis of Albracht and Bakker [34] it was proposed that NADH:Q oxidoreductase is a dimer in which one-half of the enzyme could only react with Q_{10}, whereas reoxidation by Q_{10} of the other half

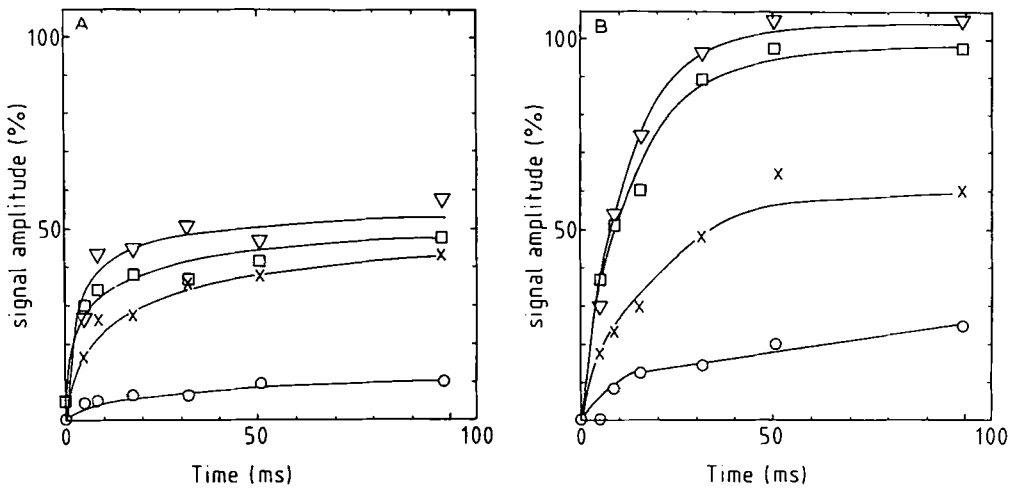

Fig. 6. Kinetics of the reduction by NADPH at pH 8.0 and pH 6.0 under aerobic conditions of the four Fe-S clusters in NADH:Q oxidoreductase present in Q_{10}-free submitochondrial particles. (A) Q_{10}-free submitochondrial particles dissolved in 0.25 M sucrose/100 mM Tris-HCl/1 mM butadione (pH 8.0) were mixed with 10 mM NADPH dissolved in the same medium. (B) Q_{10}-free submitochondrial particles dissolved in 0.25 M sucrose/100 mM Mes-KOH/1 mM butadione (pH 6.2) were mixed with 10 mM NADPH. The symbols are the same as in Fig. 2.

could only occur at low pH (pH 6–6.5). This proposal was checked by a reoxidation experiment combined with a pH jump from pH 8.0 to pH 6.3 in a three-syringe experiment. First, a pulse of NADH was used for complete reduction of all Fe-S clusters at pH 8.0, and after 1000 ms the pH was changed from pH 8.0 to pH 6.3. In this first second (before the drop in pH) half of the clusters were already reoxidised very fast (Fig. 7). After the pH change, the second half of the Fe-S clusters reoxidised slowly (Fig. 7C). This behaviour closely resembled that at a constant pH of 6.2. Both at pH 8.0 and at pH 6.2 reoxidation of all clusters 1, and half of the clusters 2 and 4, was complete within 1 s. Reoxidation of the remainder of the clusters 2 and 4 was slow (Figs. 7A and B) but it can be seen (Fig. 7B) that a low pH accelerated this reoxidation, especially that of cluster 4.

Discussion

Since trypsin treatment greatly deteriorates the stability of the NADH and NADPH oxidation activity of submitochondrial particles (Fig. 1), we preferred butadione treatment to inhibit the transhydrogenase activity. Butadione treatment had no effect on the stability of the particles at pH 6.2 and the absolute NAD(P)H oxidation activities at pH 8.0 and pH 6.2 were hardly affected (Table I). With these butadione-treated submitochondrial particles we repeated the experiments of Bakker and Albracht [33,34]. The results of our rapid-mixing rapid-freezing experiments both at pH 6.2 and at pH 8.0 differed from their report. This suggests that trypsin-treatment of submitochondrial particles induced undesired changes in the NADH:Q oxidoreductase, that affected its kinetic behaviour.

Experiments with butadione-treated Q_{10}-free submitochondrial particles at pH 8.0 and pH 6.2 (Fig. 6) strongly resembled the reduction of normal submitochondrial particles in the presence of rotenone (Figs. 2 and 4B). This is in line with the idea that rotenone inhibits the reoxidation of the Fe-S clusters by Q_{10} [46]. However, it was interesting to see whether removal of Q_{10} would also influence the kinetics of reduction of Fe-S clusters, since some authors have hinted at the possible existence of a Q cycle in Complex I [43,44]. Since NADH was still able to reduce all Fe-S clusters of NADH:Q oxidoreductase within 5 ms and NADPH reduced the Fe-S clusters in a similar way as in normal particles (in the presence of rotenone) we think it is unlikely that the pathway of reducing equivalents within this enzyme is dependent on the presence of Q_{10} as it is in Complex III, in which Q_{10} extraction does alter the kinetics of reduction because of the presence of a Q cycle, though the presence of some Q_{10} was still suspected [47] (in our case a maximum of 0.12 nmol of Q_{10} per mg of protein could still be detected). Thus, as compared to Complex III no difference was observed that could be attributed to a Q cycle in Complex I. The differences that were observed were caused by imperfect inhibition by rotenone of the reoxidation of the Fe-S clusters by Q_{10} in the pre-steady-state experiment. It has been observed earlier that when sufficient rotenone is present to inhibit NADH oxidation beyond detection, the copper signal of cytochrome *c* oxidase in submitochondrial particles still diminishes partially after addition of NADH [48]. We could confirm this observation and found that in normal rotenone-inhibited submitochondrial particles the partial decrease in the copper signal was complete within 400 ms in rapid mixing rapid-freezing experiments. We therefore conclude that

some electrons do reach ubiquinone during the pre-steady state before the inhibition becomes complete, which would indicate that rotenone is a more efficient inhibitor for the enzyme in the reduced state. We observed that no electrons could reach cytochrome *c* oxidase in Q_{10}-free submitochondrial particles. This is the reason why we consider the Q_{10}-free submitochondrial particles to be the best representative of completely inhibited Complex I for pre-steady-state experiments. The fact that the oxidation activities could not be fully restored by re-incorporation of Q_{10} was probably not caused by destruction of the NADH:Q oxidoreductase but by the fact that it is not possible to fully re-incorporate Q_{10} [38] which normally has a stronger effect upon NADH:Q oxidoreductase than upon succinate dehydrogenase. The change in NADH-ferricyanide activity upon Q_{10} extraction has been noticed earlier by Dooijewaard and Slater [49] and been explained by assuming that Q_{10} is an effector for NADH:Q oxidoreductase. The original NADH-ferricyanide activity was not restored by re-incorporation of Q_{10}, which can be related to the incomplete re-incorporation of Q_{10} (Fig. 5). Also the change in relaxation behaviour of Fe-S cluster 2, as can be observed in its EPR spectrum, was not restored by re-incorporation of Q_{10}. A similar difference in relaxation behaviour of cluster 2 could be observed by us in the EPR signals of the Fe-S cluster 2 of purified high-molecular-weight (type-1) NADH dehydrogenase, but not in purified NADH:Q oxidoreductase (Complex I) (unpublished observation). It is our opinion that this might be caused by the low amount of phospholipids in the former enzyme. Since cluster 2 is thought to be responsible for the reduction of Q_{10}, contacts between cluster 2 and phospholipids have been suggested before [50]. Extraction of Q_{10} also removes some phospholipid from the mitochondrial membrane [38]; these phospholipids were present during the re-incorporation of Q_{10}, but were apparently not re-incorporated [50].

At pH 6.2 and in the presence of rotenone both the Fe-S clusters 2 and 4 were about 80% reduced within 30 ms (Fig. 4B). In ubiquinone-free submitochondrial particles this reduction was more complete, as Q_{10}-extraction is more efficient in stopping electron transfer at the level of cluster 2. This indicates that NADPH is able to reduce these Fe-S clusters at a rate that is sufficient for these clusters to be fully involved in the NADPH oxidation activity of Complex I (the inverse turnover number

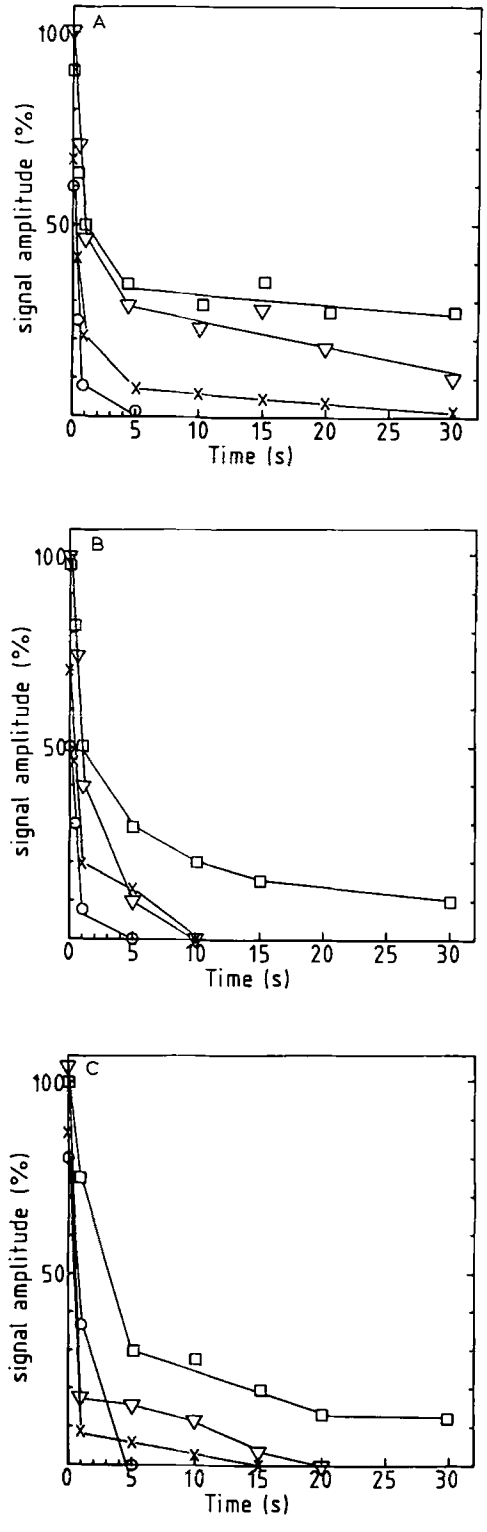

Fig. 7. Kinetics of the reduction and reoxidation of the four Fe-S clusters of NADH:Q oxidoreductase present in submitochondrial particles after addition of a small amount of NADH (75 μM) at pH 8.0, pH 6.2 and at pH 8.0 followed by a pH jump to pH 6.3 1000 ms after addition of NADH. (A) Submitochondrial particles dissolved in 0.25 M sucrose/100 mM Tris-HCl (pH 8.0) were mixed with 150 μM NADH dissolved in the same medium. (B) Submitochondrial particles dissolved in 0.25 M sucrose/100 mM Mes-KOH (pH 6.2) were mixed with 150 μM NADH dissolved in the same medium. (C) In a three-syringe experiment syringe I contained submitochondrial particles dissolved in 0.25 M sucrose (pH 8.0), syringe II contained 150 mM NADH dissolved in 0.25 M sucrose (pH 8.0), and syringe III contained a solution of 0.25 M sucrose/300 mM Mes-KOH (pH 6.2). The tubing connecting mixing chamber I with mixing chamber II was calibrated for a reaction time of 1000 ms for all points, except $t = 0$ for which a reaction time of 29 ms was taken. The symbols used were the same as in Fig. 2.

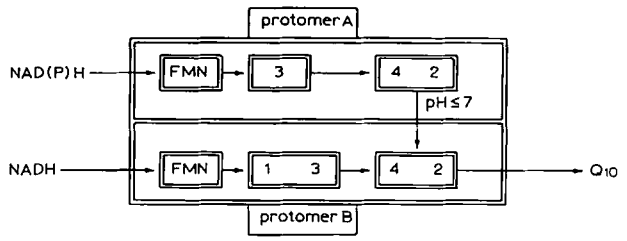

Fig. 8. Schematic representation of the oxidation and reduction reactions carried out by NADH:Q oxidoreductase under various conditions. The arrows indicate the proposed routes of electron transfer within the enzyme.

was calculated to be 30–34 ms for the enzyme). This is in conflict with the working hypothesis of Albracht and Bakker [33,34] in which NADPH is only able to reduce half of the Fe-S clusters 2 and 4. However, also the experiment reported here indicates that it is unlikely for cluster 1 and half of the clusters 3 to be involved in the NADPH oxidation activity, as is clear from Figs. 4B and 6B. In the absence of inhibitor (Fig. 4A) we observed that clusters 2 and 4 were only half reduced, while clusters 1 were hardly reduced and clusters 3 only reached about 30% reduction. The difference between Figs. 4A and B can only be explained by the fact that in the absence of an inhibitor half of the clusters 2 and 4 are rapidly reoxidised by Q_{10} at a rate greater than that at which NADPH is able to reduce these clusters. This might mean that only half of the Fe-S clusters 2 are able to react with Q_{10} at pH 6.2, which was also suggested by Albracht and Bakker [34], and is in accordance with the evidence that cluster 2 is heterogeneous in redox reactions in bovine heart mitochondria [51].

Our observations are summarised in a new working hypothesis shown in Fig. 8. Complex I is proposed to consist of a protomer A containing FMN and the Fe-S clusters 2–4 in equal amounts, and a protomer B containing FMN and the Fe-S clusters 1–4 in equal amounts (cluster 1 is completely reducible by NADH and is often referred to in literature as cluster 1b). NADPH is only able to transfer its reducing equivalents to the flavin of protomer A, while NADH is able to transfer its reducing equivalents to the flavins of both protomer A and protomer B. There is a route for transfer of electrons from protomer A to promoter B at low pH (pH 6.2) at the level of clusters 2 and 4; this route is not operational at 8.0. Furthermore, only the Fe-S cluster 2 of protomer B is able to react with Q_{10}. This working hypothesis is not in contradiction with the photo-affinity labelling experiments of Chen and Guillory [52], with which they proved that an azido analogue of NAD^+ is able to inhibit both NADH and NADPH oxidation activities of Complex I. Unfortunately, their azido-analogue of $NADP^+$ was unable to inhibit either activity. A dimeric structure of Complex I is in accordance with the hydrodynamic properties of the enzyme [49]. We again want to stress the point that the low reduction levels of clusters 1 and 3 with NADPH as substrate cannot be caused by auto-oxidation as has been proposed by Hatefi and Bearden [26], since both anaerobic and aerobic conditions led to the same results at pH 6.2 and pH 8.0 (Figs. 2 and 4). We conclude that cluster 1 and half of the clusters 3 are not involved in the NADPH oxidation activity of Complex I, since even under anaerobic conditions and in the presence of rotenone at pH 6.2 as well as at pH 8.0, NADPH needs more than 30 s to reduce clusters 1 and 3 completely (data not shown).

At pH 8.0, both in the absence and in the presence of rotenone, reduction of the Fe-S clusters by NADPH gave the same results (Fig. 2). The Fe-S clusters 2 were only half reduced and the clusters 3 and 4 even less, while the clusters 1 were hardly reduced at all. This would explain why there was no NADPH oxidation reaction: only the clusters in protomer A are reduced and no reaction with Q_{10} is possible. The fact that the clusters 3 and 4 were less than half reduced is not well understood; in Q_{10}-free submitochondrial particles they did become half reduced (Fig. 6). The extremely slow reoxidation at pH 8.0 of half of the clusters 2 and 4 after a pulse of NADH (Fig. 7A) would be explained by this scheme. Although the scheme predicts a rapid reoxidation at pH 6.2, this was not observed (Fig. 7B). Also a pH jump, applied after 50% reoxidation of the clusters 2, speeded up the reoxidation of the second half of the clusters 2 (Fig. 2C). This was indistinguishable from the reoxidation behaviour of the Fe-S clusters at pH 6.2 (Fig. 7B and C).

How reoxidation of the clusters 2 actually takes place is not completely understood. Fe-S clusters are one-electron donors and ubiquinone needs two electrons to become fully reduced. During NAD(P)H oxidation by Complex I in submitochondrial particles semiquinones (that could be associated to Complex I) were never observed in any of our experiments. This would mean that the reduction of semiquinones to quinols is very rapid. This seems to be in contrast with the finding of Q_{10}-binding proteins in Complex I that would stabilise semiquinones [43,53].

The effect of NAD^+ at pH 8.0 is partly caused by the non-enzymic reduction of NAD^+ by NADPH, which is slow but sufficient to form a detectable amount of NADH under the experimental circumstances (Fig. 3A and B). In order to prevent this non-catalytic NADH formation, NAD^+ was added to the submitochondrial particles before mixing with NADPH (Fig. 3C). In this case a more rapid reduction of clusters 2 and 4 was observed, while cluster 1 was hardly reduced. The rapid reduction of half of the clusters 2 and 4 was followed by further reduction at a moderate rate, an effect not seen in the absence of NAD^+ (Fig. 2B). When rotenone was not added, the reduction of the first half of the clusters

was still slightly faster than in the absence of NAD^+ (data not shown), but the slow phase of reduction was not observed probably due to reoxidation. That reoxidation did take place was confirmed in an activity assay where addition of NAD^+ together with NADPH resulted in slight oxidation of NADPH at a rate comparable to that predicted from the slow phase of reduction seen in the pre-steady-state experiment (Fig. 3C). This might be ascribed either to an NADPH-NAD^+ transhydrogenase activity that is still active even though no NADPH-$AcPyAD^+$ activity could be detected, or to stimulation of the transfer of reducing equivalents from the clusters 2 and 4 in protomer A to those in protomer B. No stimulating effect of NAD^+ could be detected at pH 6.2. In fact, addition of 2 mM NAD^+ to submitochondrial particles and 5 mM NADPH lowered the rate of oxygen consumption of this mixture as measured by a Clark electrode (data not shown).

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