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Hypothesis

Proton-translocating transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenases operate in a substrate cycle which contributes to fine regulation of the tricarboxylic acid cycle activity in mitochondria

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Abstract

H^+-transhydrogenase (H^+-Thase) and NADP-linked isocitrate dehydrogenase (NADP–ICDH) are very active in animal mitochondria but their physiological function is only poorly understood. This is especially so in the case of the heart and muscle, where there are no major consumers of NADPH. We propose here that H^+-Thase and NADP–ICDH have a combined function in the fine regulation of the activity of the tricarboxylic acid (TCA) cycle, providing enhanced sensitivy to changes in energy demand. This is achieved through cycling of substrates by NAD-linked ICDH, NADP-linked ICDH and H^+-Thase. It is proposed that NAD–ICDH operates in the forward direction of the TCA cycle, but NADP–ICDH is driven in reverse by elevated levels of NADPH resulting from the action of the transmembrane proton electrochemical potential gradient (Δp) on H^+-Thase. This has the effect of increasing the sensitivity to allosteric modifiers of NAD–ICDH (NADH, ADP, ATP, Ca^{2+} etc), potentially giving rise to large changes in the net flux from iso-citrate to α-ketoglutarate. Furthermore, changes in the level of Δp resulting from changes in the demand for ATP would, via H^+-Thase, shift the redox state of the NADP pool and this, in turn, would lead to a change in the rate of the reaction catalysed by NADP–ICDH and hence to an additional and complementary effect on the net metabolic flux from isocitrate to α-ketoglutarate. Other consequences of this substrate cycle are, (i) the production of heat at the expense of Δp, which may contribute to thermoregulation in the animal, and (ii) an increased rate of dissipation of Δp (leak).

Key words: Mitochondrion; Tricarboxylic acid cycle; Regulation; Futile cycle

1. Introduction

H^+-transhydrogenase (H^+-Thase) in the inner membranes of animal mitochondria catalyses the transfer of hydride ion equivalents between NAD(H) and NADP(H) coupled to the translocation of protons,

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

where ${H^+}_{out}$ and ${H^+}_{in}$ signify the involvement of protons on the cytosolic and matrix side of the membrane, respectively. The catalytic site for oxidation and reduction of the nicotinamide nucleotides is exposed on the matrix side. Thus, in principle, H^+-Thase can either consume the proton electrochemical gradient (Δp) generated by the respiratory chain in order to drive the reduction of mitochondrial $NADP^+$ by NADH or, during reduction of NAD^+ by NADPH, it can generate Δp to supplement that derived from respiration. For reviews of the structure and mechanism of H^+-Thase (see [1–3]).

Animal mitochondria have two matrix-located iso-citrate dehydrogenases, one NAD-linked and the other NADP-linked,

$$IC + NAD^+(NADP^+) \leftrightarrow KG + NADH(NADPH) + CO_2 \quad \text{Equ. (2)}$$

The equilibrium constant of the reaction is 1.17 M [4]. For reviews, see [5,6].

2. Earlier proposals for the function of ICDH and H^+-transhydrogenase

There is a clear consensus view that flux through IC to KG in the forward direction of the TCA cycle is

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Abbreviations: TCA, tricarboxylic acid; IC, isocitrate; KG, ketoglutarate; Δp, transmembrane proton electrochemical potential gradient; H^+-Thase, H^+-transhydrogenase; ICDH, isocitrate dehydrogenase.

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carried predominantly by NAD–ICDH [7–10]. The view is consistent with the manner in which the enzyme is regulated: it is activated by Ca^{2+}, ADP, and citrate and inactivated by ATP, NADH and NADPH [11].

In contrast, the functions of NADP-linked ICDH and of H^+-Thase have remained uncertain. However, it has often been recognised that (i) the functions of NAD–ICDH, of NADP–ICDH and of H^+-Thase should be considered together and, (ii) the activities of NADP–ICDH and H^+-Thase are highly significant relative to those of other enzymes of the TCA cycle and oxidative phosphorylation. Thus, the activity of NADP–ICDH (in the forward direction) in the mitochondrial matrix is some *ten times* greater than that of NAD–ICDH [8–10], and the rate of H^+-Thase in energised submitochondrial particles is only a little less than the rate of ATP synthesis [12].

An early suggestion ([13], discussed in [8–10]) was that substantial TCA cycle flux might proceed through NADP–ICDH and that the resulting NADPH would be oxidised by the respiratory chain via H^+-Thase. It was supposed that the proton-pumping activity of the latter would contribute to the generation of Δp (hence H^+-Thase was described as 'Site 0' or 'Loop 0' of the respiratory chain). However, this possibility was discounted on the basis of flux measurements with isolated mitochondria [9,10] and experiments with an inhibitor, methyl-isocitrate, that is specific for NADP–ICDH [8]. Transhydrogenase activity from NADPH to NAD^+ in coupled rat liver mitochondria was less than 10% of the rate of respiration [9] and methyl-isocitrate inhibited isocitrate oxidation by only about 20% [8]. With hindsight, this suggestion seems inherently unlikely. The idea that NADH and NADPH would both be generated by ICDH, that NADH, of course, would also be generated by other dehydrogenases of the TCA cycle, and that the two nucleotides would donate electrons to the respiratory chain at two diferent levels across a coupling site would require very complex control mechanisms and it is difficult to imagine what advantage would be gained.

A development of this hypothesis was put forward by Hoek and Rydstrom [14]. They proposed that, during brief periods of energy depletion, H^+-Thase might utilise existing levels of NADPH to drive outward proton translocation and therefore prevent Δp from falling to low values: H^+-Thase was envisaged as a Δp 'buffer'. Although we should not like to discount this hypothesis – it has a number of attractive features – there are two drawbacks. First, the H^+/H^- ratio for H^+-Thase (x in Equ. (1)) is probably only in the range 0.5–1.0 [2,15]; the enzyme is not a very good proton pump. This is reflected, for example, in the fact that it is difficult to drive ATP formation by transhydrogenation from NADPH to NAD^+ even in well-coupled submitochondrial particles [16]. Note that in contrast to H^+-Thase, which translocates only 0.5–1.0 H^+ per $2e^-$ equivalents, respiratory activity from NADH to $1/2O_2$ results in the translocation of about 10 H^+ [17]. Second, the capacity of the NADPH pool in the mitochondrial matrix is rather low. Based on an estimated mitochondrial content of about 2 nmol/mg protein and a rate of respiration of about 100 nmol/min/mg protein [11], it can be calculated that the NADPH would last for less than 0.5 s before complete exhaustion.

A contrasting view is that H^+-Thase and NADP–ICDH function in the synthesis of intramitochondrial NADPH to meet particular metabolic needs. There might be some substance to this proposal, particularly in liver, where there is a clear requirement for NADPH in a number of mitochondrial reactions, including the reductive amination of ammonium [18] and the reduction of damaging hydroperoxides via glutathione reductase and glutathione peroxidase [19,20] (discussed in [1,21,22]). Hoek and Rydstrom [14] have developed a more sophisticated version of this hypothesis. They argued that H^+-Thase can serve, not only as a 'Δp buffer' (see above), but it can also operate to buffer the nucleotide levels (particularly NADPH) in the mitochondrial matrix. Thus, when there is a sudden increased demand for NADPH, it can be satisfied by Δp-driven H^+-Thase. It remains to be explained why H^+-Thase and NADP–ICDH are both required as NADPH generators, particularly as the activity of the latter (in the forward direction) considerably exceeds that of the former (see below). Another difficulty for the idea that H^+-Thase and NADP–ICDH are important in NADPH generation is that some mitochondria, notably from heart and muscle, which have high activities of these enzymes [9,14], have a very low biosynthetic capacity. While it is recognised that NADPH will still be required within these mitochondria for the reduction of glutathione to minimise damage from free radical production in the respiratory chain, it seems unlikely that large quantities of the reduced nucleotide will be consumed for this purpose. The rate of production of free radicals is, at most, 1–9% of the respiration rate of animal tissues and is normally somewhat less than this [21,50], whereas the rate of $NADP^+$ reduction by H^+-Thase and NADP–ICDH is potentially two to three orders of magnitude greater. And again, in the context of this hypothesis, the question arises as to why there should be two NADPH generators.

In tissues such as liver there is a much higher demand for NADPH in the cytosol (for fatty acid biosynthesis, mixed function oxidation, etc.) than in the mitochondrial matrix. It has, therefore, been suggested that the function of H^+-Thase might be to transfer reducing equivalents from NADH (presumably derived from the oxidation of fatty acids, or from reversed electron transport [22]) to NADPH in the mitochondrial matrix for export to the cytosol via an IC–KG shuttle [22,23]. NADPH would be consumed in the matrix of the mitochondria by NADP–ICDH operating in reverse, and then regenerated in the cytosol by the equivalent enzyme functioning in the for-

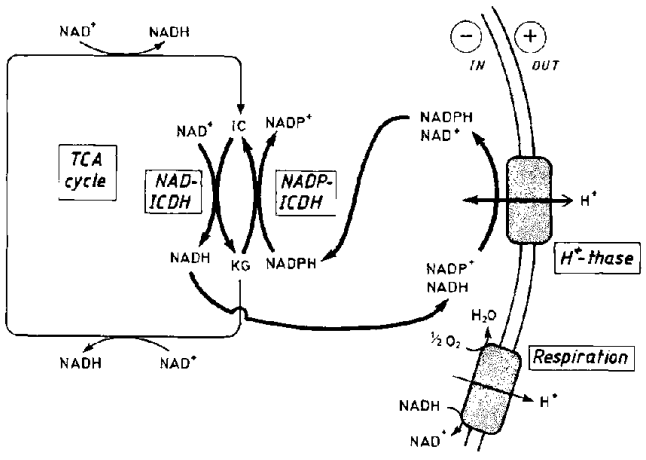

Fig. 1. The scheme of the proposed substate cycle through NAD–ICDH, H^+-thase and NADP–ICDH (bold lines).

ward direction. The tricarboxylic acid transporter, catalysing the export of citrate and IC, and the dicarboxylic acid transporter, catalysing the import of KG, complete the process. This pathway is entirely plausible for liver mitochondria. However, because of the low biosynthetic capacity of heart and muscle, it is unlikely to be important for mitochondria from these tissues. Furthermore, the activity of tricarboxylic acid transporters [24,25] in heart mitochondria, and of NADP–ICDH in the cytosol of heart cells [26,27] are very low.

In summary, earlier hypotheses for the function of H^+-Thase and NADP–ICDH might be partly correct when applied to liver but they do not adequately explain the high activities of these enzymes in heart and muscle. In this discussion, we leave aside the functions of H^+-Thase and ICDH in bacteria and in yeast; it is possible that they are quite different to those in animal cells. The consensus view is that, at least in some bacteria, H^+-Thase and NADP–ICDH both provide NADPH for cellular biosynthesis, but see [28 31] for detailed observations. Yeast mitochondria seem not to possess H^+-Thase [54], but do have both NAD- and NADP-linked ICDH; the former is thought to carry TCA cycle flux, the function of the latter is unknown [55].

3. Substrate cycles

It is widely accepted that the existence of a substrate cycle in animal cells between fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate (F1,6P), catalysed by phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (F1,6Pase), provides increased sensitivity in the control of flux through the glycolytic pathway [32,33]. It is evident that there are circumstances in which both enzymes operate in unison at the expense of continued hydrolysis of ATP. The purpose is ostensibly to amplify the effect of changes in the concentration of AMP (an allosteric effector) on the net rate of phosphorylation of F6P. Thus, regulation of the net flux in the direction of glycolysis becomes more sensitive as the rate of the reverse reaction approaches that of the forward reaction [33]. The consumption of ATP is the metabolic 'price' to pay for the improved level of control. Additionally, at least in some animals, the heat generated from this substrate cycle seems to be important in thermoregulation [32,34]. Other substrate cycles (e.g. glucose/glucose-6-phosphate, pyruvate/phosphoenolpyruvate) have also been proposed to be involved in metabolic regulation [32,33,35,52]. There are three minimum conditions for the operation of a substrate cycle; (i) the presence of two different enzymes for the same metabolic step, (ii) reversibility, and (iii) that the system must be ultimately driven by energy [35].

4. A Δp-driven substrate cycle between IC and KG mediated by transhydrogenase

There appear to be many factors contributing to the control of the TCA cycle and oxidative phosphorylation in mitochondria, not all of which are clearly understood (reviewed [7,36–38]). ICDH is an important control point [9,11,36]. Studies of metabolite concentrations in heart mitochondria revealed that the reaction catalysed by NAD–ICDH is sufficiently removed from equilibrium to exert its regulatory role [9] and the enzyme is known to be subject to complex allosteric regulation by a variety of effectors (see above). There are no known allosteric effectors of NADP–ICDH.

We propose (Fig. 1) that, in the mitochondrial matrix, a substrate cycle operates between IC and KG. In accordance with current opinion [7], NAD–ICDH operates in the direction IC --> KG but the reverse reaction is catalysed by NADP–ICDH. NADPH as substrate for the reverse reaction is supplied by H^+-Thase driven by Δp. The net reaction is simply the dissipation of Δp. Thus,

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

$$IC + NAD^+ \rightarrow KG + NADH + CO_2$$

$$KG + NADPH + CO_2 \rightarrow IC + NADP^+$$

$$SUM \quad xH^+_{out} \rightarrow xH^+_{in}$$

This device provides a means by which net flux through the TCA cycle is (a) more sensitively controlled by the allosteric modifiers of NAD–ICDH, and (b) directly controlled by the energy state of the inner mitochondrial membrane (Δp). We suggest that, under most circumstances, to ensure sensitive control, the rate of the forward, NAD–ICDH-catalysed reaction is only slightly

greater than the rate of the reverse, NADP–ICDH-catalysed reaction.

Under conditions in which the demand for mitochondrial ATP is low, the consequent elevation of the ATP and lowering of the ADP concentrations, together with the increase in the NADH and decrease in the NAD^+ concentrations resulting from 'respiratory control', will all combine, through allosteric interaction, to decrease the rate of NAD–ICDH [11,36]. According to the principles discussed at length in [32,33], the decrease in rate of NAD–ICDH will have a more pronounced effect on net flux from IC to KG when NADP–ICDH is performing the reverse reaction at a substantial rate. For example, suppose that, before the decrease in ATP demand, the rate of the reaction catalysed by NAD–ICDH is 100 units and the rate of the reverse reaction catalysed by NADP–ICDH is 50 units (the net flux is 50 units). If, after decreasing the ATP demand, the allosteric effectors cause a decrease in the rate of NAD–ICDH by 40%, then the net flux from IC to KG will be decreased by five-fold to 10 units.

As a consequence of the substrate cycle, there may be another significant effect, of thermodynamic origin, of decreasing ATP demand on the net flux from IC to KG. Following a decrease in ATP demand, the resulting change in ΔG for ATP hydrolysis (it will become more negative) will cause the F_oF_1-ATP synthase to decrease in rate. Then, as Δp is no longer consumed at a high rate, its value will rise, and this will have the effect of driving the transhydrogenase reaction to the right, in favour of NADPH formation. In turn, the elevated concentration of NADPH will result in an increase in the rate of reductive carboxylation of KG to IC catalysed by NADP–ICDH. It will be suggested below that this is expected to be only a small change in rate. However, the effect will be amplified by the operation of the substrate cycle. Thus, using the figures in the example above, an increase in the rate of reverse NADP–ICDH by only 10% will further decrease the net flux from IC to KG by a factor of two.

In a complementary way, in moving from a state of low to high ATP demand, the amplifying properties of the substrate cycle will result in a large increase in the flux from IC to KG as a result of allosteric modification of NAD–ICDH and the thermodynamic effect on NADP–ICDH. In working heart, large changes in workload (5–10-fold) are accompanied by only very small changes of ATP and ADP concentrations in mitochondria [39]. Substrate cycling between IC and KG might therefore provide a mechanism which would allow flux through the TCA cycle to follow precisely the changes in energy demand and thus match immediately the changes in energy consumption and production.

It is now well established that Ca^{2+} is an important activator of the dehydrogenases of the TCA cycle, including NAD–ICDH [7,38]. The concentration of Ca^{2+}

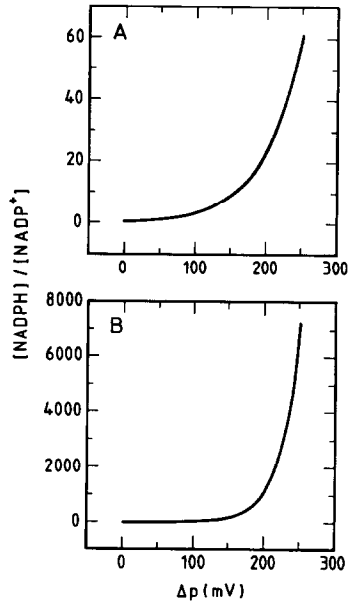

Fig. 2. The equilibrium relationship between Δp and [NADPH]/[$NADP^+$] ratio for the transhydrogenase reaction according to Equ. (3). [NADH]/[NAD^+]=0.5, H^+/H^- ratio equals 0.5 (A) and 1.0 (B).

in the cytoplasm and hence in the mitochondrial matrix is regulated by hormones and other messengers. Possibly, in muscle tissues, regulation of the activity of the TCA cycle by external signals via Ca^{2+} is more primary than the response to changes in ATP demand. The sensitivity of net flux from IC to KG to changes in the concentration of Ca^{2+} will be increased by the operation of the ICDH substrate cycle in an equivalent manner to that described above. Interestingly, Denton et al. [46] reported that NAD–ICDH in mitochondria is considerably more sensitive to activation by Ca^{2+} than the isolated enzyme in free solution (the K_a in the intact organelle is about 10 times less than that in solution). This observation is neatly explained by the substrate-cycling hypothesis. Furthermore, an additional site of regulation of the ICDH cycle by metal ions might be H^+-Thase. As we reported recently, this enzyme is strongly activated by low concentrations (in the physiological range) of Ca^{2+} and Mg^{2+} [47].

The energy cost of the ICDH substrate cycle is somewhat less than that of an ATP-driven cycle. Only 0.5–1.0 H^+ (x, above) are returned across the inner mitochondrial membrane for one complete turn of the ICDH cycle compared, for example, with 1.0 ATP hydrolysed for the PFK/F1,6Pase system. Since probably $3H^+$ are translocated through the ATP synthase for the synthesis of one ATP in the mitochondrial matrix, and the equivalent of one more proton translocation reaction is required to transfer the ATP to the cytosol [17], the energy cost to turn the ICDH cycle is 1/8 to 1/4 of that for the PFK/F1,6Pase cycle. This is presumably a reflection of the larger free energy change (under physiological conditions) accompanying the conversion of F6P to F1,6P by

PFK than that accompanying the conversion of IC to KG by NAD–ICDH.

It is evident from this description that the proposed ICDH substrate cycle would operate in addition to mechanisms of control of the TCA cycle that are already well documented. By analogy with arguments that have been raised in the context of the F6P/F1,6P substrate cycle [32,33], it would serve to increase the sensitivity of flux through a major control point in the TCA cycle. A precise quantitative analysis of the rates of the component reactions and of the variations of flux with the energy state of the mitochondria is presently not possible. However, we will show below that the operation of the cycle is at least plausible, even likely, based on what is known about the properties of the isolated enzymes and the concentrations of metabolites in isolated mitochondria.

5. Experimental data consistent with a transhydrogenase/Δp-driven substrate cycle through iso-citrate dehydrogenase

5.1. Directionality of NAD- and NADP–ICDH in vivo

The concentrations of IC and KG in isolated heart/liver mitochondria are in the range 0.02–0.2 and 0.2–2 mM, respectively [9,11,40]. The CO_2 concentration in vivo is about 1.5 mM [53]. Determination of the concentrations of the free oxidised and reduced nicotinamide nucleotides in the mitochondrial matrix is extremely difficult because substantial fractions are bound to proteins. The use of 'indicator metabolites' is a valuable procedure to measure in vivo concentrations of nucleotides, provided that the reaction catalysed by the relevant enzyme can be shown to be at equilibrium, usually by repeating with a second (and third) enzyme [42]. Unfortunately, it has been difficult to do this exhaustively in the case of the mitochondrial nucleotides. Given this reservation, the matrix NAD pool is generally thought to be mainly oxidised (E_h = −280 to −320 mV) and the NADP pool highly reduced (E_h = −390 to −415 mV) [11,14,40]. Thus, from the published equilibrium constant of 1.17 M [4], the ΔG for the reaction catalysed by NAD–ICDH is approximately −10 to −16 kJ·mol^{-1} and the reaction is expected to proceed from IC to KG. Because the redox potential of the NADP pool is known less precisely, there can be less confidence for NADP–ICDH, but ΔG is likely to be in the range −1 to +2 kJ·mol^{-1} and we conclude from this that under in vivo conditions it is quite conceivable that this enzyme does function in reverse. For example, if the matrix concentrations of IC, KG and CO_2 were 0.03, 0.9 and 1.5 mM, respectively, the reaction would tend to proceed from KG to IC if the E_h of the NADP pool were less than −373 mV, at pH 7.4. It will be shown below (Fig. 2) that at transhydrogenase equilibrium, when the E_h of the NAD(H) pool is −320 mV, the E_h of the NADP(H) pool will fall below −373 mV when Δp is more than 103 or 206 mV (depending on whether x in Equ. **(1)** is 1.0 or 0.5, respectively). These values of Δp are within the range that has been recorded experimentally [17].

A case can be made that, whereas the kinetic properties of NAD–ICDH are consistent with it operating in vivo only in the direction IC → KG, the properties of NADP–ICDH indicate that it can function in either direction. Dalziel [51] observed that 'mitochondrial NAD–ICDH's show positive rate cooperativity with respect to isocitrate. They are effectively unidirectional catalysts, unlike the NADP-linked enzymes (presumably because their K_m values for CO_2 are large)'. In fact, the K_m for CO_2 of NADP–ICDH, at high but non-saturating concentrations of the other reactants, is 1.6 mM [41], i.e. in the region of the concentration of CO_2 in vivo (1.5 mM). Furthermore, the affinity of NADP–ICDH for NADPH is about 100 times greater than for $NADP^+$ [43], which would also fit with its operating from KG to IC. Interpretation of kinetic data for the carboxylic acid substrates is rather complicated, due to the complex effects of divalent cations and uncertainties over their in vivo concentrations, but the enzyme 'binds IC very strongly and KG rather weakly' [43]. However, it will be discussed below that, at concentrations of IC and KG reported for the mitochondrial matrix, the reaction in vitro can proceed rapidly in the direction from KG to IC.

5.2. Energetics of the transhydrogenase reaction in the context of the directionality of ICDH

At equilibrium, the mass action ratio of the nucleotide substrates of the transhydrogenase reaction is related to the proton electrochemical gradient by the following equation:

$$\Delta p = (RT/xF)\ln[NADPH][NAD^+]/[NADP^+][NADH] \quad \text{Equ. (3)}$$

where x is the H^+/H^- ratio. Fig. 2 shows the equilibrium relationship between Δp and the [NADPH]/[$NADP^+$] ratio, for x = 0.5 and 1.0, assuming that the [NADH]/[NAD^+] ratio is 0.5. The real value of x is expected to lie between 0.5 and 1.0, the limits set by experiment [2,15]. It is evident that, through the action of transhydrogenase, the NADP pool would be predominantly reduced over the range of Δp that is usually estimated in isolated mitochondria (180–200 mV [17]) and that, thermodynamically, for measured concentrations of IC and KG of 0.02–0.2 and 0.2–2 mM [9,11,40], respectively, the redox potential of the $NADP^+$/NADPH would be low enough to drive NADP–ICDH from KG to IC (see above).

Fig. 2 illustrates that the relationship between the [NADPH]/[$NADP^+$] ratio and Δp is nonlinear. An increase in Δp of 20 mV predicts an increase in [NADPH]/

[$NADP^+$] at equilibrium by a factor of 1.5 to 2.1, dependent on x. In principle, such a change could have a kinetic effect on the rate of the reaction catalysed by NADP–ICDH, i.e. an increase in Δp would increase the rate of reaction from KG to IC. Though the effect would be quite small (probably, at most a factor of 2.1 for a 20 mV increase in Δp, if x were 1.0), through substrate cycling this could have a large inhibitory effect on the *net* flux from IC to KG, depending on the relative rates of the forward (NAD-linked) and reverse (NADP-linked) ICDH (see above).

5.3. *The catalytic capacity of the enzymes involved in the putative ICDH substrate cycle*

It is difficult to measure the activities of H^+-Thase and NAD- and NADP-linked ICDH in intact cells or even in isolated mitochondria. An examination of the rates of the component reactions from the literature indicated to us that, on the basis of its kinetic competence, the ICDH substrate cycle is at least plausible. Recently, during the development of the hypothesis described above, we carried out an analysis of the rates of H^+-Thase, NAD–ICDH, NADP–ICDH and State 3 and 4 respiration in appropriate fractions of the same mitochondrial preparation. Specific activities were within the ranges that have been measured by others but the results were normalised relative to the protein concentration of the intact organelles [12]. Although it is difficult confidently to extrapolate from these results to the in vivo situation, the rates of NAD–ICDH operating in the forward direction, of NADP–ICDH operating in the reverse direction and of H^+-Thase operating in the forward direction, with respect to the measured rate of State 4 respiration, were consistent with the operation of a significant ICDH substrate cycle. The following should be considered. (i) Because in vivo the enzyme is subject to pronounced allosteric control, the in vitro rate of NAD–ICDH cannot be considered very critically in the context of the ICDH substrate cycle. Even so, there are no obvious discrepancies with the hypothesis. (ii) When reverse NADP–ICDH was measured in the presence of concentrations of IC (0.05–0.1 mM) and KG (0.4–2 mM) that have been reported for the mitochondrial matrix, the rate was only 15–30% less than the maximum rate recorded in the absence of products [12] and, thus, in principle, could support substrate cycling. (iii) Forward H^+-Thase was measured with thio-$NADP^+$ as substrate. On the basis of [12], this analog is an adequate substitute for transhydrogenase reaction, although the rate with the physiological substrate could be somewhat higher. (iv) The transhydrogenase reaction was measured in submitochondrial particles energised by succinate respiration and in the absence of products. The rate is expected to decrease to some extent with increasing concentrations of NADPH and NAD^+. However, it seems that under these conditions the forward reaction of H^+-Thase is relatively insensitive to product inhibition at least until the concentration of product increases to very high levels. According to [45], the energy-linked reaction proceeds with an almost linear rate until the NADPH/$NADP^+$ ratio exceeds a value of approximately 50. Furthermore, it may be noted that, because a large fraction of the mitochondrial NADPH is enzyme bound, the concentration of free NADPH might be very low, even at a very low redox potential of the nucleotide. Thus product inhibition of H^+-Thase is not expected to affect critically the rate of reaction.

5.4. *Evidence for reversibility of ICDH in isolated mitochondria*

In support of their view on the export of reducing equivalents as NADPH from liver mitochondria (see above), Hoek and Ernster [23] and later Wanders et al. [22] described experiments which might indicate the in vivo reversibility of NADP–ICDH. They found an increase in the rate of citrate export from intact liver mitochondria upon elevation of the concentration of bicarbonate in the medium. The increase was inhibited by uncoupling agents. The results can also be considered to support our hypothesis of an ICDH substrate cycle driven by Δp and mediated by H^+-Thase but, unfortunately, they do not provide unequivocal evidence of *net* reversal of NADP–ICDH. Thus, even if NADP–ICDH were operating (close to equilibrium) in the forward direction, its rate would be decreased through a mass-action effect by an increase in the CO_2 (bicarbonate) concentration; the resulting increase in IC and citrate would lead to an increase in their export rate.

In isolated liver mitochondria there is an experimental observation that is in conflict with the suggestion (above) that NADP–ICDH operates 'in reverse', from KG to IC. Thus, treatment with methyl-isocitrate, a specific inhibitor of NADP–ICDH, led to a decrease, not an increase, in the amount of matrix NADPH [8]. This might indicate that we should indeed confine our hypothesis to heart and muscle mitochondria (see above). However, it has to be pointed out that there are experimental difficulties associated with the use of methyl-isocitrate. First, although the ratio of the K_m for IC/K_i for methyl-isocitrate is approximately 100 [8] and the in vivo concentration of IC is in the range 0.02–0.2 mM [9,11,40], concentrations of methyl-isocitrate as high as 0.4–0.7 mM decreased the state 4 NADPH concentration by only 20% [8]. Admittedly, the contribution to NADPH production by transhydrogenase under these conditions is not known, which further complicates the situation. Second, methyl- isocitrate chelates Mg^{2+} and therefore has an indirect inhibitory effect on NAD–ICDH. The presence of high external Mg^{2+} concentrations was intended to minimize this effect [8] but the Mg^{2+} permeability of the inner mitochondrial membrane is rather low [38].

That the NADP–ICDH in vivo should operate in re-

verse is a critical prediction of our hypothesis and further experimental work in this area would be valuable.

6. Other consequences of the ICDH substrate cycle

For each turn of the ICDH substrate cycle, between 0.5 and 1.0 H^+ (dependent on x) are taken up across the inner mitochondrial membrane. This will make some contribution to the membrane proton 'leak', thought to account for as much as 30% of the net respiratory rate even in mitochondria in intact cells [34,48]. The quantitative significance of H^+ uptake accompanying the ICDH substrate cycle is difficult to assess; it will depend on flux round the substrate cycle relative to net flux through the TCA cycle. If, for example during a period of low ATP demand, the ICDH substrate cycle operates at about ten times the rate of the net flux through the TCA cycle (as in the example in section 4, this being compatible with the data described [12]), then (assuming that the H^+/O ratio is 10 and 6 for NADH and succinate, respectively [17]) it can be calculated that in the region of 10–20% of the respiratory-driven proton efflux will return through the H^+-Thase. In principle, if Δp were generated to higher levels, especially in view of the non-linear behaviour illustrated in Fig. 2, leak through this system could be of much greater significance.

It has been pointed out [32] that the two roles of substrate cycling, amplification and heat generation, are intimately linked and should be discussed together. The clearest indication of the importance of a substrate cycle in heat generation is the PFK/F1,6Pase cycle in the flight muscle of the bumble-bee [32]. In mammals, the main sources of heat generation under resting conditions are thought to be the metabolically active internal organs, such as liver [34,49]. About 25–40% of total heat produced is attributed to the processes accompanying oxidative phosphorylation (including proton leak) and the rest to ATP-consuming processes, which have not been precisely identified [34]. Amongst these, there may be a significant contribution by various substrate cycles [32,33,52], for example up to 50% of the basal metabolic rate was attributed to PFK/F1,6Pase cycle in some vertebrates [32]. In active animals, a higher proportion of heat is produced in the muscles. In newborn and hibernating animals a large contribution to total heat generation is made by the thermogenin-based substrate cycle involving the circulation of protons across the membranes of brown fat mitochondria [34,49]. Proton leak in mitochondria other than those from brown fat was also suggested to be involved in heat production in animals [34].

Following the arguments in [32], it is quite conceivable that the ICDH substrate cycle proposed above has a role in thermogenesis, but the quantitative significance can not be assessed with information available at the present time.

7. Conclusion

There seems to be a good case to consider that a 'futile' or 'substrate' cycle, involving H^+-Thase, driven by Δp, and NAD- and NADP–ICDH, serves to regulate the TCA cycle in animal mitochondria. The properties of the isolated enzymes and the available information on substrate concentrations in the matrix of isolated mitochondria and relevant thermodynamic parameters, indicate the feasibility of the process. The ICDH substrate cycle might also have a role in thermogenesis.

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