REGULATION OF ENERGY METABOLISM: EVIDENCE AGAINST A PRIMARY ROLE OF ADENINE NUCLEOTIDE TRANSLOCASE

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1. Introduction

Measurements in suspensions of isolated mitochondria respiring in the presence of substrate and oxygen [1] as well as in suspensions of cells [2–4] and in perfused organs [5,6] show that the first two sites of mitochondrial oxidative phosphorylation are at near equilibrium. This is expressed in the equation:

$$NADH + 2cyt\ c^{3+} + 2ADP + 2P_i \rightleftharpoons NAD^+ + 2cyt\ c^{2+} + 2ATP \quad (1)$$

where NAD^+ and NADH represent the intramitochondrial concentrations of the free coenzymes and ATP, ADP and P_i represent the extramitochondrial concentrations of the adenine nucleotides and inorganic phosphate.

It is well established that the intramitochondrial [ATP]/[ADP][P_i] measured in vitro in state 4 [7–9] and in suspensions of cells in vivo [10,11] is 10–100-fold smaller than that in the extramitochondrial space. Therefore if mitochondrial oxidative phosphorylation involves net inward flow of ADP from the extramitochondrial to intramitochondrial space and a net flow of ATP in the opposite direction then this movement of ADP^{3-} in and ATP^{4-} out (the adenine translocase reaction) occurs against an apparent concentration gradient and must require metabolic energy. It has been postulated that the energy needed for the adenine translocase reaction arises through the operation of the respiratory chain which generates a membrane potential, negative inside, sufficient to drive the electrogenic exchange of the adenine nucleotides [7,12]. Thus according to this postulate, 20–30% of the total free energy used to maintain the extramitochondrial [ATP]/[ADP][P_i] is derived through translocation (equiv. approx. 3 kcal) and only 70–80% by way of the ATP synthesis per se in the mitochondria. This yields a stoichiometry of approx. 1.6 ATP synthesized in the extramitochondrial space per NADH oxidized in reaction 1 which would affect calculations of the free energy relationships carried out assuming a stoichiometry of 2 for the same reaction.

Paracoccus denitrificans offers a direct means of attaining information on the stoichiometry of ATP synthesis. This microorganism has a respiratory chain which closely resembles that of mammalian mitochondria [13] and because it is unicellular and prokaryotic, partitioning of cellular components and compartmentation of reactants within the cell is unlikely. All the ATP is therefore synthesized in what is probably a single intracellular space and active transport of the adenine nucleotides (translocase reaction) is not an intermediate in the phosphorylation reaction.

This paper describes the results of studies on the free energy relationships between the redox reactions of the respiratory chain and ATP synthesis in cell suspensions of *P. denitrificans*. The data show that in this microorganism reaction (1) is near equilibrium as found [1–6] in other types of cells.

Moreover, the measured relationship of cellular respiration to intracellular [ATP]/[ADP][P_i] and [NAD^+]/[NADH] fits the quantitative model for the regulation of mitochondrial respiration developed [14] for mammalian mitochondria. It is concluded that

mitochondrial adenine nucleotide translocation affects neither the free energy relationships in reaction 1 nor the regulation of respiration.

2. Materials and methods

Paracoccus denitrificans was grown aerobically at 30°C in a medium containing (per liter): K_2HPO_4, 6.0 g; KH_2PO_4, 4.0 g; NH_4Cl, 1.0 g; vitamin-free casamino acids (Difco) 5.0 g; glucose, 20 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $MnSO_4 \cdot 4H_2O$, 0.001 g; $CaCl_2$, 0.04 g; $Na_2MoO_4 \cdot 7H_2O$, 0.15 g; $FeSO_4 \cdot 7H_2O$, 5.5 mg; and citric acid 5.25 mg [15].

Cultures were harvested in the logarithmic phase of growth and washed twice in 50 mM phosphate, pH 7.0. The washed cells were suspended in 50 mM phosphate buffer containing 20% sucrose and incubated for 20 min at room temperature. The cells were then washed twice in Krebs-Henseleit saline containing 10 mM Tris–HCl (pH 7.4) instead of bicarbonate.

2.1. *Incubations*

The cells (approx. 5 mg dry wt/ml) were incubated in Krebs-Henseleit–Tris saline, pH 7.4, containing 10 mM glucose as the substrate for 8–10 min under oxygen with rapid shaking. At the end of the incubation period, the cells were either quenched by the addition of cold perchloric acid (3% final conc.) or used for the determination of the redox state of cytochrome *c* and the respiratory rate. The perchloric acid extracts were neutralized with 3 M K_2CO_3–0.5 M triethanolamine base mixture and aliquots of the extracts were used for assays of metabolites (see below). P_i was measured in parallel experiments in which the cells were rapidly separated from the suspending medium by centrifugation (Eppendorf centrifuge 5412) through a layer of silicone oil (Versilube® F 50, General Electric, Silicone Products Dep. Waterford, NY). The intracellular water content of the cell was determined for each preparation [16].

2.2. *Measurement of the redox state of cytochrome* c

A sample of the incubated cell suspension was added to a spectrophotometric cuvette in a Johnson Foundation dual wavelength spectrophotometer and the redox state of cytochrome *c* was measured using the wavelength pair 550–540 nm. In order to prevent sedimentation the suspension of cells was continuously mixed with a vibrating stirrer. After the initial reading had become stable, 50–100 μM antimycin A was added to cause complete oxidation of cytochrome *c*. The observed decrease in concentration of reduced cytochrome *c* was taken to correspond to 100% oxidation of this cytochrome. The fully reduced state of cytochrome *c* was taken to be that after the addition of 2 mM cyanide. The redox state of cytochrome *c* was calculated as in [2,3] using an $E_{m7.0}$ value of 230 mV [17].

2.3. *The respiratory rate*

Respiratory rate calculated as turnover number for cytochrome *c* (electrons/s) was determined from the oxygen uptake measurements (Clark-type O_2 electrode) at 22°C with 10 mM glucose as the respiratory substrate.

2.4. *Measurements of the [NAD⁺]/[NADH] ratio*

The cellular [NAD^+]/[NADH] ratio in suspension of cells was calculated as in [18] from the 3-OH butyrate and acetoacetate concentrations measured as in [19]. The activity of 3-OH-butyrate dehydrogenase was 12.4 μmol NAD reduced/h/mg protein of broken cells. The equilibrium constant for the reaction at 25°C and pH 7.0 was taken to be 1.4×10^{-2} [20].

2.5. *Measurements of ATP, ADP and P_i*

ATP was determined as in [21] ADP as in [22] and P_i as in [23].

2.6. *Protein*

This was measured using the biuret reaction with bovine serum albumin as a standard.

2.7. *Reagents*

Analytical grade reagents were all obtained commercially. Enzymes were all the products of Sigma (St Louis, MO).

3. Results

Table 1 summarizes the experimental results necessary for the calculation of the equilibrium

Table 1
Free energy relationships between the redox reactions of the respiratory chain and the phosphorylation state of the adenine nucleotide system in suspensions of *Paracoccus denitrificans*

Reactant or reactant ratio		
$\frac{[3\text{-OH-Butyrate}]}{[\text{acetoacetate}]}$	0.18 ± 0.013	(4)
$\frac{[NAD^+]}{[NADH]}$	397 ± 31	(4)
$\frac{[\text{cyt } c^{3+}]}{[\text{cyt } c^{2+}]}$	5.91 ± 0.79	(7)
ATP	4.09 ± 0.44	(6)
ADP	0.54 ± 0.07	(6)
P_i	4.02 ± 0.25	(3)
$[ATP]/[ADP][P_i]$ (M^{-1})	1880 ± 125	(3)
TN (e^-/s/cyt *c*)		
E_h cyt *c* (V)	0.276	
E_h NAD (V)	−0.244	
ΔE_h	0.520	
ΔG_{ox-red} (kcal/2e^-)	−24.0	
ΔG_{ATP} (kcal/2 ATP)	24.11	
$\Delta G_{ox-red} - \Delta G_{ATP}$ (kcal)	0.11	

The E_m of cytochrome *c* was taken to be 0.230 V [17] and that of [3-OH-butyrate]/[acetoacetate] as −0.266 V [20], at pH 7.0 and 25°C

The *Paracoccus denitrificans* cells were obtained and treated as described in the text. ATP, ADP and P_i are calculated as μmol/ml intracellular water. Values are expressed as means ± SEM for the number of experiments in parentheses. Each estimation was carried in duplicate. The free energy relationships are calculated for two phosphorylation sites.

$E_h = E_h([NAD^+]/[NADH]) - E_h([\text{cyt } c^{3+}]/[\text{cyt } c^{2+}])$ where

$$E_h = E_m \frac{2.3\,RT}{nF} \log \frac{ox}{red}; \quad \Delta G_{ox-red} = -nF\Delta E;$$

$$\Delta G_{ATP} = \Delta G'_o + 1.36 \log \frac{[ADP][P_i]}{[ATP]}$$

relationships between the respiratory chain and the phosphorylation state of the adenine nucleotide in suspensions of *P. denitrificans* cells. All the calculations were made for pH 7.0 which was within 0.1 pH unit of the value measured in the incubation mixture at the end of the incubation time. The ratio of [3-OH-butyrate]/[acetoacetate] was about 0.2 very similar to that in isolated liver cells and cultured kidney cells incubated without added substrate [2,3] and in free-living ciliate protozoon *Tetrahymena pyriformis* [4]. This, free $[NAD^+]/[NADH]$ calculated from the equilibrium constant of the 3-OH-butyrate dehydrogenase reaction was approx. 400.

The 3-OH-butyrate dehydrogenase activity of *P. denitrificans* cells was high: 12.4 μmol NAD reduced/h/mg protein of broken cells which is over 5-fold greater than the activity found in *T. pyriformis* cells [4]. The activity of glutamic dehydrogenase was less than 1 μmol NAD reduced/h/mg protein and therefore this enzyme was not a suitable indicator of the cellular NAD couple. (NADP-dependent glutamic dehydrogenase was 4.58 μmol/h/mg protein of broken cells, which suggests the glutamic dehydrogenase may provide reducing equivalents for biosynthetic purposes.) The levels of intracellular lactate were too low to determine the redox state of the NAD couple from the equilibrium in the lactate dehydrogenase reaction.

The ATP, ADP and P_i concentrations were similar to those found in other types of cells [1–6]: 4 mM, 0.5 mM and 4 mM, respectively. The [ATP]/[ADP]-values were about 8 and the $[ATP]/[ADP][P_i]$values approached 2000 M^{-1}.

Cytochrome *c* was approx. 18% reduced which is very close to the value found in liver cells (15–20% reduced) in the absence of added substrate [2,3] and slightly less than in kidney cells (25%) [24] or *T. pyriformis* cells (30–35%) [4]. The respiratory rate expressed as turnover number for cytochrome *c* was 13.5 e^-/s which is very similar to the turnover number for cytochrome *c* in kidney cells [24], *T. pyriformis* cells [4] and perfused heart under 'normal' work load conditions [6] but higher than in the liver cells in the absence of added substrate [2,3].

From the data presented in table 1, the free energy changes associated with the transfer of 2 reducing equivalents from the NAD to cytochrome *c* couple and the free energy change required for ATP synthesis can be calculated. It follows from the E_h values of

cytochrome *c* and the NAD couple that the difference of E_h (ΔE) between the two couples is 0.520 V. The corresponding free energy change for transfer of 2 reducing equivalents from NADH to cytochrome *c* is −24.01 kcal. The Gibbs free energy change associated with the synthesis of 2 mol ATP can be calculated from the measured [ATP]/[ADP][P_i] and $\Delta G'_o$ of ATP hydrolysis of 7.6 kcal/mol [25]. The value is 24.11 kcal/2 ATP. From the comparison of the values for ΔG of oxidation–reduction and ΔG for ATP synthesis, it can be seen that the difference between the two is within an experimental error, equal to zero.

Essentially the same results were obtained on suspensions of spheroplasts prepared from *P. denitrificans* cells and are not given here.

4. Discussion

4.1. *Free energy relationships between the oxidation–reduction reactions and phosphorylation*

The experimental results reported in this work demonstrate that in suspensions of the bacterium *P. denitrificans* a near-equilibrium exists between the redox-reactions of the respiratory chain from the NAD to cytochrome *c* couples and the intracellular [ATP]/[ADP][P_i]. These calculations rest on the assumption that there are 2 phosphorylation sites between the NAD and cytochrome *c* and that the transfer of 2 reducing equivalents across the sites results in synthesis of 1 mol ATP. Although the experiments reported here do not provide any direct information on the number of sites or electron pathway they do show, however, that there is sufficient free energy to synthesize 2 mol ATP during oxidation of NADH by cytochrome *c*. The validity of this assumption is further justified by the fact that the respiratory chain of *P. denitrificans* closely resembles that of mammalian mitochondria with respect to its structural and functional properties [13] as well as by the observation that sites 1 and 2 were found to be present in heterotrophically grown cells of this microorganism [26]. The NADH-linked substrates are oxidized at high rates and the redox-components of sites I and II including the iron–sulfur proteins (T. K., T. Salerno, T. Ohnishi and T. Lillich, in preparation) have similar thermodynamic characteristics (half-reduction potential values) as those in mammalian or avian mitochondria (reviewed [27]).

The conclusion of a near-equilibrium in the first two sites of oxidative phosphorylation in *P. denitrificans* not only confirms and extends our previous results obtained on other types of cells [1–4,6,24] but further strengthens them because of the inherent simplicity of the system. For any practical purpose, *P. denitrificans* can be considered as a single compartment. Mesosomes, if present, do not appear to contain ATPase coupling factor nor are major sites of oxidative reactions [28]. Therefore the membrane-bound respiratory chain utilizes ADP and P_i and synthesizes ATP which are all freely exchangeable in a single compartment (intracellular space) and not segregated within various cell organelles. Under such conditions there is only one [ATP]/[ADP][P_i] pool and the question of the differences between the extramitochondrial and intramitochondrial phosphorylation potential is not relevant.

The results reported here demonstrate that in *P. denitrificans* where no translocation of adenine nucleotides is required during oxidative phosphorylation the same free energy relationships are observed as in cells in which the ATP synthesized in the mitochondrial matrix has to be moved to the cytosol. Thus, if the intramitochondrial [ATP]/[ADP][P_i] in the latter cells is indeed different from that of the extramitochondrial space [7–11] more thought should be given to the possibility (e.g. [29]) that under phosphorylating as well as dephosphorylating conditions the adenine nucleotide translocator and the mitochondrial ATPase can be functionally linked to catalyze phosphorylation or dephosphorylation of extramitochondrial ADP or ATP without equilibration with the intramitochondrial adenine nucleotides.

It has been suggested [30,31] that the ATPase activity of *P. denitrificans* is not reversible and that respiratory rate is controlled kinetically at the ATPase step. This suggestion was based on experiments performed with isolated membrane fragments which showed that under the particular experimental conditions [30,32] the ATPase activity was 10% or less of the rate of ATP synthesis. Unfortunately, the range of experimental conditions used for the ATPase assay was rather limited and not necessarily optimal. (The effects of pH, ionic strength and the time-course of ATP hydrolysis were not investigated.) Especially in

state 3 but also in state 4 for these particles, oxidative phosphorylation is highly irreversible, with a large negative free energy change (ΔG) in the direction of ATP synthesis. It is not unusual for enzymes to have different measured maximum velocities in the forward (ATP synthesis) and reverse directions (ATP hydrolysis) because of different assay conditions and different driving forces involved. The observation that the ΔG of the coupled reaction approaches zero (this work) provides evidence for the reversibility of the ATPase reaction in vivo.

4.2. *Regulation of cellular energy metabolism*

A model for the reaction of cytochrome *c* oxidase has been proposed [14] and combined with reaction (1) to give a mathematical expression which correctly predicts the relationships among the intramitochondrial $[NAD^+]/[NADH]$, the extramitochondrial $[ATP]/[ADP][P_i]$ and the mitochondrial respiratory rate [4]. If reaction (1) is near equilibrium and the rate constants for the *P. denitrificans* cytochrome *c* oxidase are similar to those for the mammalian enzyme this mathematical expression should also fit the data for *P. denitrificans* cells. In intact cells, free Mg^{2+} concentration is approx. 1 mM [33] and all of the equilibrium constants involving ATP and ADP must be appropriately corrected. The values for these corrected constants used for the fitting are given in table 1.

The relationships between the respiratory rate

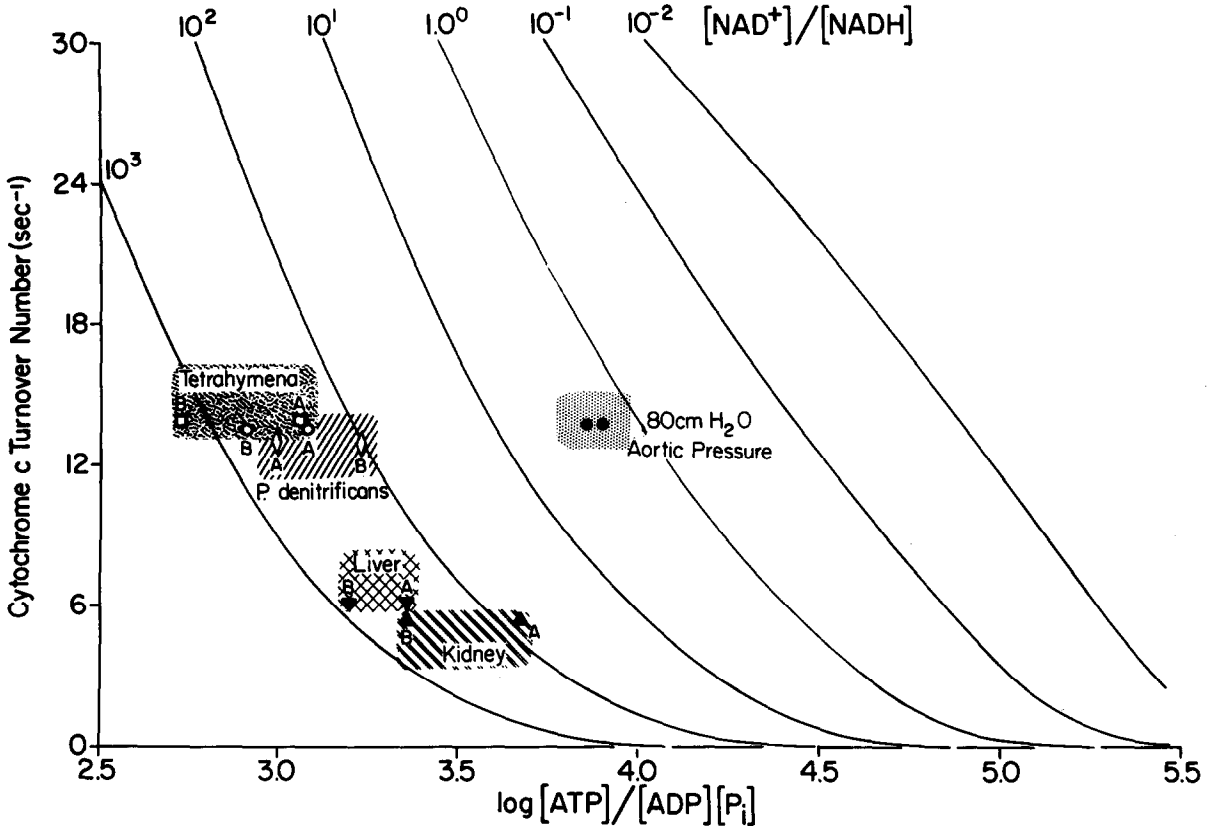

Fig.1. Fit of experimental results to the mathematical model of regulation of mitochondrial respiration. Solid lines are plotted according to [4,14] while experimental results are plotted as two points. Data points labeled A represent the turnover number of cytochrome *c* plotted against the intramitochondrial $[NAD^+]/[NADH]$ while data points labeled B represent the cellular $[ATP]/[ADP][P_i]$ plotted against the turnover number of cytochrome *c*. The points for perfused heart isolated liver cells and *Tetrahymena pyriformis* cells are taken from [4] and those for cultured kidney cells from [24]. Shaded areas indicate range of values between points A and B.

(turnover number for cytochrome *c*) and the cellular $[ATP]/[ADP][P_i]$ calculated according to the model are plotted on the same figure which results effectively in a three-dimensional graph. The experimental results must be represented by two points: point A, where the measured turnover number for cytochrome *c* is plotted versus the measured $[ATP]/[ADP][P_i]$ and point B, where the measured turnover number for cytochrome *c* is plotted against the measured $[NAD^+]/[NADH]$. Superposition of the two points indicates an ideal fit of the data by the mathematical model. Figure 1 shows the results obtained for *P. denitrificans* (this work) as well as those for isolated liver cells, cultured kidney cells and rat heart perfused by the Langendorff technique with 80 cm aortic pressure taken from [4,6]. The data points A and B fall very close to one another in each experimental system which indicates that the mathematical expression is equally successful in fitting the regulation of energy metabolism in *P. denitrificans* as it is in mammalian cells.

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