CHAPTER 21

A THERMODYNAMIC ANALYSIS OF DICARBOXYLIC ACID PRODUCTION IN MICROORGANISMS

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21.1 INTRODUCTION

The production of chemicals from renewable feedstocks using microorganisms has received considerable attention due to the ever increasing possibilities of genetic engineering and the potential to decrease CO_2 emissions. Multifunctional molecules containing alcoholic and carboxylic acid groups are especially of interest, because of their application in polymer production which represents a very large market [1]. Four carbon 1,4-diacids (succinic, fumaric, and malic) are amongst the top 12 chemical building blocks manufactured from bio-feed-stocks in a report by the US Department of Energy (USDOE) [2].

As a consequence, the biotechnological processes will occur on a very large scale where, from a cost point of view, one desires the highest product yield on a particular substrate, low cost of auxiliary chemicals (e.g., *p*H control), and low cost of downstream processing (DSP). A general approach to evaluate these aspects, leading to relevant targets for genetic engineering, therefore is of interest and is the focus of this contribution. The approach will be illustrated using two dicarboxylic acids (succinic and fumaric acid) and will be thermodynamically-based, ensuring its general applicability to any other product of interest.

21.2 OUTLINE OF THE APPROACH

For dicarboxylic acids, one has to consider several aspects:

- There is production of acid which requires the consumption of alkali for *p*H control in the fermentation process. In DSP, the conventional method used to obtain undissociated acid (needed in the final polymerization processes) requires the addition of an inorganic acid solution. Overall, this leads to the consumption of stoichiometric amounts of alkali (e.g., $CaCO_3$) and acid (e.g., H_2SO_4) leading to the production of stoichiometric amounts of salt (e.g., $CaSO_4$). Purchase of the alkali and acid and disposal of the salt pose a significant cost factor of about € 0.30 per kg acid (see Appendix 21.A.1). Economically, it is desirable to perform the fermentation process at low *p*H, producing the undissociated acid which avoids the need for these auxiliary agents.
- The dicarboxylic acids are produced in metabolic reaction networks which require that the product is exported over the membrane. While this is no problem for alcohols (passive diffusion is possible), intracellular organic acid anions (charge –2) require special transporters which must achieve the required large out/in acid concentration ratio (typically on the order of 10^3; intracellular concentrations of dicarboxylic acids are usually in the range of 10^{-3} mol/L and an economically viable process requires about a 1 mol/L concentration in the broth). Therefore, the export of the acid has an energy aspect. This energy aspect is even augmented, because all the produced H^+ must also be exported against the H^+ gradient (proton motive force [*pmf*]).
- Organic acids, especially at the preferred low extracellular *p*H, can only be exported using energy consuming active transport mechanisms as explained above. However, at low *p*H, the richly available extracellular undissociated organic acid can freely diffuse back into the cell, leading to an energy consuming (futile) cycle of export and import of undissociated acid. This energy drain leads to a changed product yield [3].
- At low *p*H organic acids often reach their solubility limit. Our approach uses a thermodynamic framework to analyze the production of dicarboxylic acids (succinic and fumaric) from glucose at low *p*H with respect to the stoichiometric and energy aspects of the black-box, theoretical product reaction and candidate metabolic networks.

21.2.1 Black Box thermodynamic analysis of the theoretical dicarboxylic acid product reaction

This analysis for the two dicarboxylic acids mentioned above will present the maximal theoretical yield, the theoretical product reaction, the consumption of alkali, the

osmotic strength and amount of solid product as a function of *p*H, and, finally, the calculation of $\Delta_r G$ as a function of *p*H.

21.2.2 Maximal theoretical product yield

A product made by microorganisms is the result of a metabolic network. Theoretically, the highest product yield is obtained when the organism does not spend substrate for growth, maintenance, or an external electron acceptor (such as O_2). This leads to a theoretical situation where one envisages that the cell only makes the intended product under ***anaerobic conditions***, i.e., all electrons from the substrate end in the product. The theoretical maximal yield then follows from a simple balance of degree of reduction [4]:

$$Y_{p/s}^{theor}\left(\frac{\text{moles of product (p)}}{\text{moles of subsrate (s)}}\right) = \frac{\gamma_s}{\gamma_p} \tag{21.1}$$

Assuming glucose ($C_6H_{12}O_6$, $\gamma_s = 24$) as a renewable substrate and considering succinic acid ($C_4H_6O_4$, $\gamma_p = 14$) or fumaric acid ($C_4H_4O_4$, $\gamma_p = 12$) as products, the values of $24/14 = 1.71$ and $24/12 = 2.00$ as maximal theoretical molar yields for succinic and fumaric acid per mole of glucose are obtained. This theoretical maximal yield would be achieved anaerobically and is not based on any metabolic reaction network assumption. In principle, this black box result poses a theoretical maximum for any conceivable network which starts with glucose and produces the acid as the only product. However, the feasibility of this yield needs to be checked from a thermodynamic point of view, but first the stoichiometry of the theoretical product reaction is required.

21.2.3 Stoichiometry of the theoretical product reaction

A dicarboxylic acid in aqueous solution is composed of 4 different species which are undissociated acid dissolved in water (aq) due to a limited solubility (see Fig. 21.1) there is solid undissociated acid (s) present and the dissolved undissociated acid dissociates at increasing *pH* into its mono (−1) and di(−2) anion.

Considering the four product species and using succinic acid as an example, we can write the product reaction as (note that consumed compounds have negative coefficients and that CO_2 is assumed to be in equilibrium with the gas phase):

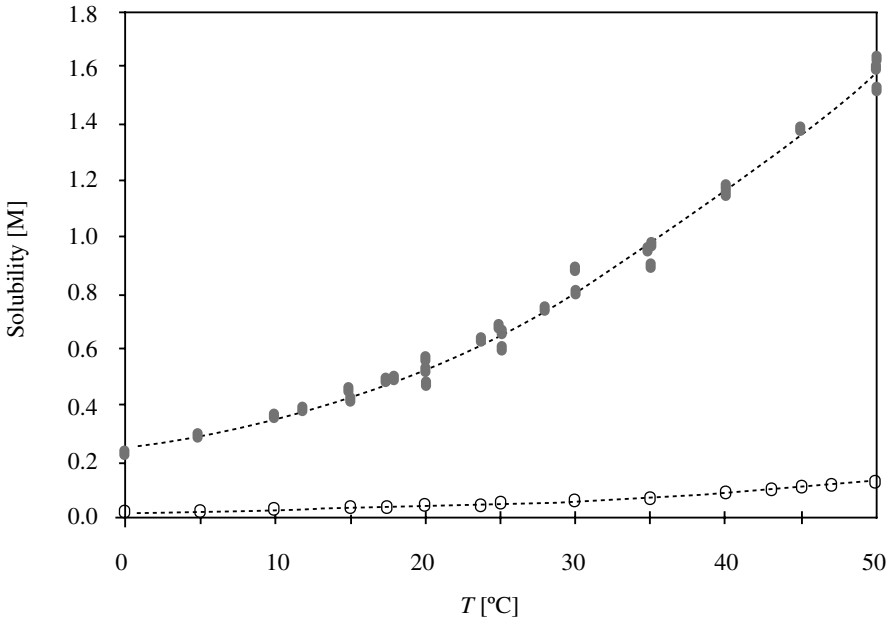

Fig. 21.1 Solubility of undissociated fumaric acid (open symbols) and succinic acid (closed symbols) [40] as a function of temperature.

$$a\,C_6H_{12}O_6(aq) + b\,CO_2(g) + c\,C_4H_6O_4\,(s) + d\,C_4H_6O_4(aq) + e\,C_4H_5O_4{}^{-1}(aq) \qquad (21.2)$$
$$+ f\,C_4H_4O_4{}^{2-}(aq) + g\,H_2O + h\,H^+$$

The 8 coefficients (a to h) can be calculated with the following two regimes.

Dissolved acid regime

In this regime, there is absence of solid product, hence $c = 0$ in Equation 21.2. The 7 stoichiometric coefficients remain to be calculated with these 7 equations:

- the 4 conservation (C, H, O, charge) equations:

$$6a + b + 4(d+e+f) = 0 \qquad (21.3a)$$

$$12a + 6d + 5e + 4f + 2g + h = 0 \qquad (21.3b)$$

$$6a + 2b + 4(d+e+f) + g = 0 \qquad (21.3c)$$

$$-e - 2f + h = 0 \qquad (21.3d)$$

- the product sum relation (we assume that the sum of all acid species equals 1 mol knowing that $c = 0$):

$$d + e + f = 1 \tag{21.3e}$$

- two dissociation equilibrium relations (the dicarboxylic acids have two dissociation equilibria with dissociation equilibrium constants of pK_1 and pK_2):

$$H_2A \leftrightarrow HA^- + H^+$$

and

$$HA \leftrightarrow A^{2-} + H^+$$

These equilibria give the following two relations when we consider a situation where the stoichiometric coefficients of the succinic species become identical to their concentrations (requiring that the sum of all organic acid is equal to 1 mol/L):

$$\frac{e}{d} = 10^{(pH-pK_1)} \tag{21.3f}$$

$$\frac{f}{e} = 10^{(pH-pK_2)} \tag{21.3g}$$

Table 21.1 shows the pK values for succinic and fumaric acid at an assumed temperature of 25°C.

The above 7 relations (Eq. 3a to Eq. 3g) can be used to obtain the 7 coefficients a, b, d, e, f, g, and h as a function of pH. The coefficient h represents the molar amount of H^+ produced per mole of total dicarboxylic acid (equal to 2 at higher pH). The coefficients d, e, and f represent the amount of each of the three organic acid species. If we assume that the fermentation process needs (for an economical DSP) a total product concentration of 1 mol/L, then d, e, and f represent the concentrations of the three acid species (dissolved undissociated, mono- and di-dissociated). The coefficient b represents the consumed CO_2 and a is the

Table 21.1 pK_1 and pK_2 values for fumaric and succinic acid dissociation at 25°C [32].

Compound	pK_1	pK_2
Fumaric acid	3.09	4.6
Succinic acid	4.21	5.64

consumed glucose. Assuming glucose limited continuous fermentation, where the residual glucose concentration can be neglected, then the coefficient a represents the concentration of glucose in the feed solution (assuming that the in and out volume flow rates are equal).

Solving the 7 equations shows that coefficients a and b are independent of pH. In addition, b follows from the C balance and a follows from the balance of degree of reduction. As shown in Equation 21.1, $a = \left(Y_{p/s}^{theor}\right)^{-1}$. The theoretical minimal glucose consumption per mol acid is: $a = 1/1.71 = 0.5833$ moles of glucose (succinic acid) and $a = 1/2.00 = 0.50$ moles of glucose (fumaric acid).

Interestingly, there is also pH independent CO_2 consumption where $b = 0.50$ and 1.00 mole of CO_2 consumed per mole of succinic and fumaric acid, respectively.

The coefficients d, e, f, g, and h depend on the pH. At high pH, $h = 2.0$, because the only acid species present has a charge of −2 ($f = 1$). At decreasing pH, the stoichiometric coefficient f (species with charge of −2) decreases monotonically, e (species with charge of −1) increases and then decreases, and d (undissociated acid) increases monotonically (see Fig. 21.2).

Solid acid regime

Below a certain pH, the concentration of undissociated acid will rise beyond its solubility (Figure 21.1). At 25 ºC for succinic acid the solubility limit (0.64 mol/L) is reached at pH = 3.95 and for fumaric acid the solubility limit (0.047 mol/L) is reached at pH = 4.11.

Below these pH-values, we enter another regime where solid acid is present. In this regime, the stoichiometric coefficient $c \neq 0$, but the concentration of the dissolved undissociated acid (d) remains constant at its solubility limit, hence now $d = 0.64$ and 0.047 for succinic and fumaric acid, respectively. The 7 equations above (Eq. 21.3) can then be solved again to obtain the other coefficients which apply to the low pH range (see Fig. 21.2). Figure 21.2 shows that, at a low pH of about 3, most (90%) of the fumaric acid is present as a solid making DSP very attractive. At low pH, most succinic acid is present as dissolved acid.

21.2.4 Alkali consumption, osmotic stress and ionic strength

The theoretical product reaction stoichiometry (Fig. 21.2) shows that when the amount of H^+ varies, then different amounts of alkali need to be added to control the pH. Usually NaOH or $CaCO_3$ is used and the cost of alkali (used in fermentation) and acid (used in DSP) is considerable, about € 0.30/kg acid (Appendix 21.A.1). The addition of NaOH to the bioreactor for pH control leads then to accumulation of Na^+ at higher pH which causes a steep increase in osmotic and ionic strength. Figure 21.3 shows the results. It appears that the increase of pH from 1 to 8 leads to a steep increase in osmotic stress with a maximum (at pH ≈ 6) osmotic strength

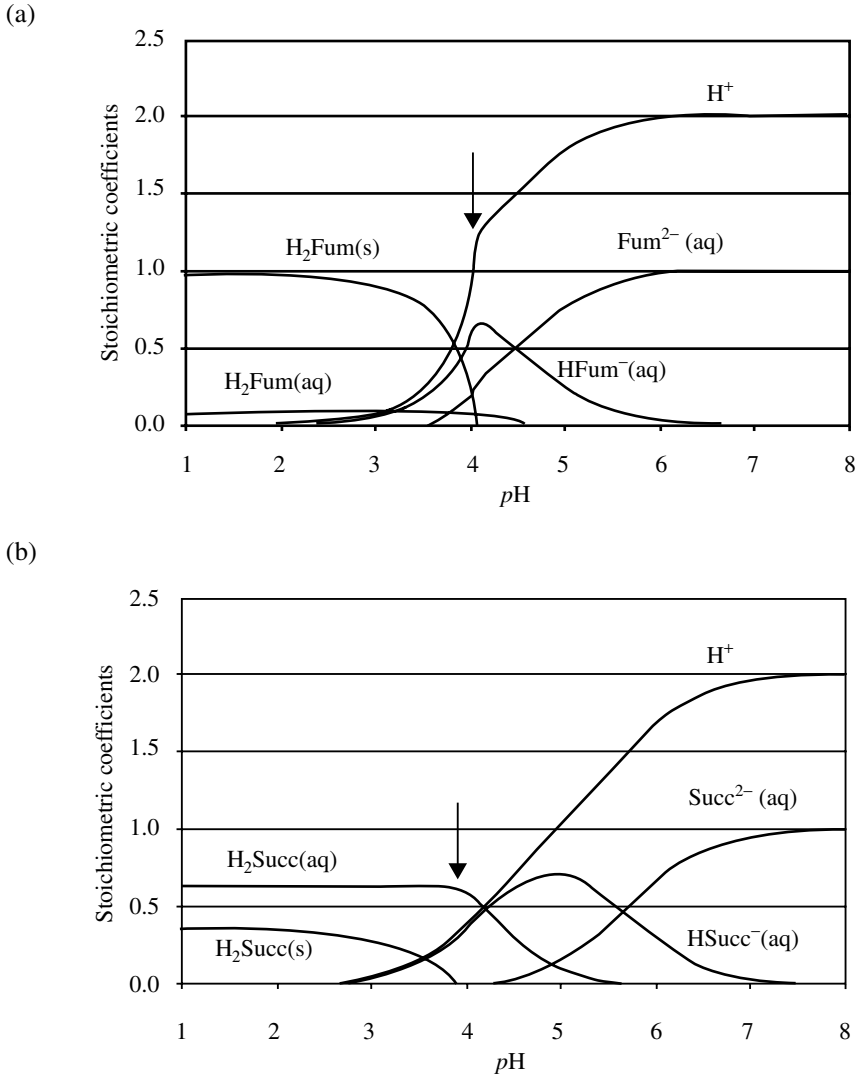

Figure 21.2 Stoichiometry of organic acid species and H^+ in the theoretical product reaction for 1 mole of total organic acid as a function of *p*H at 25 °C: (a) fumaric acid and (b) succinic acid. The arrows indicate the solubility limit.

equalling 3 mol/L. Also, the ionic strength increases and if 1 mol/L is assumed for dicarboxylic acid, then the maximal ionic strength also equals 3. Interestingly, at low *p*H (≈3) the ionic strength becomes close to zero for both acids, but the osmotic strength is different, being low (≈0) for fumaric acid (low solubility) and much higher (≈0.6 mol/l) for succinic acid.

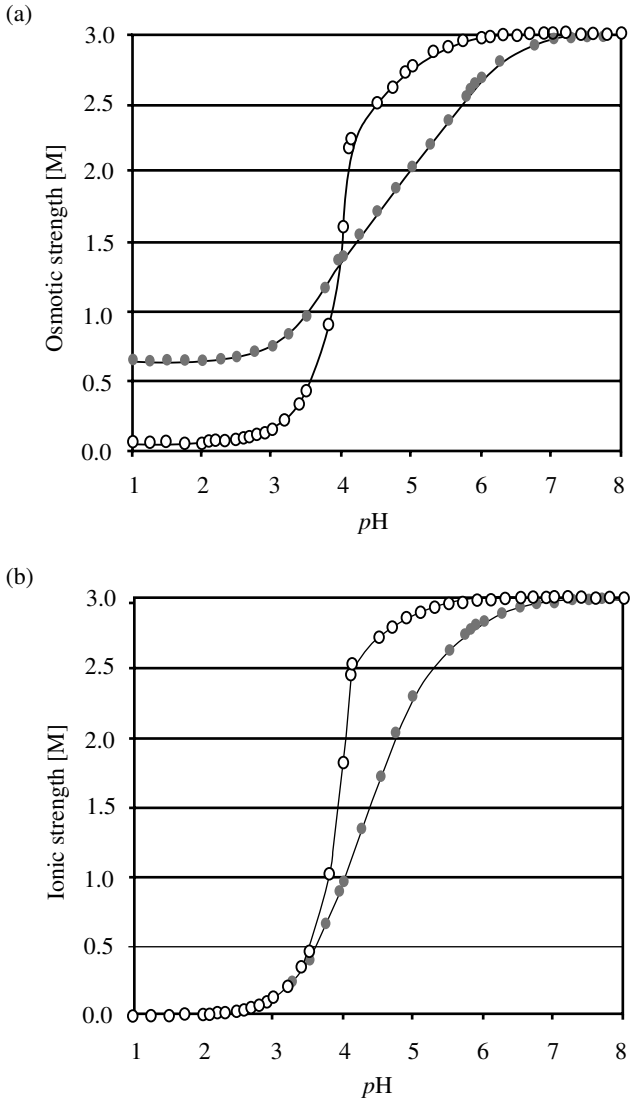

Figure 21.3 Osmotic (a) and ionic (b) strength of broth as function of *p*H for succinic acid (closed symbols) and fumaric acid (open symbols).

Clearly, at low *p*H the production is not only economically advantageous, due to the absence of alkali needed for fermentation and acid needed for DSP, but the cells are also exposed to much lower ionic/osmotic stress. Especially for fumaric acid, which has low solubility, the osmotic/ionic stress at low *p*H is marginal. It is very relevant to avoid such stresses, because they can lead to formation of unwanted by-products, e.g., glycerol [5], which lowers the production yield.

21.2.5 Thermodynamics of product formation

Having elaborated the theoretical product reaction stoichiometry as a function of *pH* (Fig. 21.2), it is possible to calculate $\Delta_r G$. Here the standard $\Delta_f G°$ values were used (25 ºC, see Appendix 21.A.2). In addition, we assume that the residual glucose concentration is 1 mmol/L (10^{-3} mol/L) and that CO_2 gas is supplied at 1 bar pressure (standard condition). Zelle *et al.* showed that a high CO_2 level is also relevant for the production of malic acid [6].

In principle, $\Delta_r G$ also depends on temperature and ionic strength. Our calculation shows that their effects are minor (only a few kJ difference, results not shown here) which agrees with the conclusion of Maskow and von Stockar [7]. Also, it was found that a 10 fold change in residual glucose only changes $\Delta_r G$ by a few percent.

Using the results of Figure 21.2, the value of $\Delta_r G$ follows from Figure 21.4 which shows three main results:

- for both dicarboxylic acids, $\Delta_r G$ (kJ/mol acid) is very negative, therefore, the theoretical, anaerobic product reaction could allow production of biochemical useful energy (ATP, *pmf*);
- $\Delta_r G$ becomes less negative with decreasing *p*H until *p*H ≈ 4, however, below *p*H ≈ 4 most of the acid is in the undissociated form and the *p*H effect disappears (clearly, the economic advantage for fermentation and DSP at low *p*H is at the expense of the energy production potential for the organism);

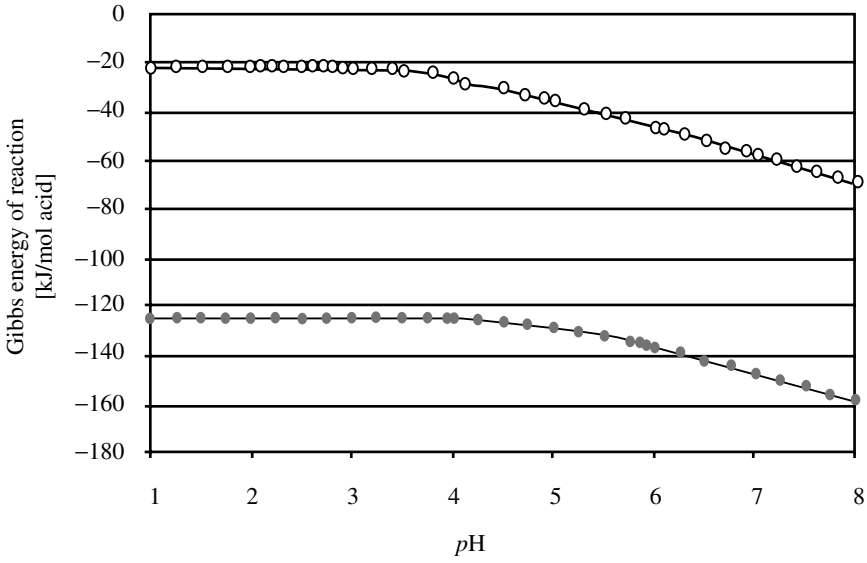

Figure 21.4 $\Delta_r G$ (kJ/mol acid) of succinic acid (closed symbols) and fumaric acid (open symbols) as a function of *p*H.

- $\Delta_r G$ for fumarate is, in the whole *p*H range, significantly less negative than for succinate, which shows that the higher efficiency of DSP with fumaric acid (solid product) occurs again at the expense of the energy production potential for the organism.

These results show a general trade-off behaviour with respect to the total available Gibbs energy, i.e., the Gibbs energy available for the organism, the Gibbs energy consequences of low *p*H (for fermentation and DSP), and the presence or absence of solid product (DSP); there is no free lunch, somebody has to pay!!

21.3 THERMODYNAMICS OF DICARBOXYLIC ACID TRANSPORT

21.3.1 Thermodynamically feasible transport mechanisms

The relevance of transport energetics for monocarboxylic acids has been reviewed already [8,9]. Here, we will focus on dicarboxylic acids. In the previous section (21.3), it has been shown for succinic and fumaric acid, even at low *p*H (≈3), that the theoretical anaerobic product reaction is thermodynamically very feasible from an overall (Black Box) point of view.

This fact means that, in principle, the proposed theoretical product reaction under anaerobic conditions could function as a sole source of biological energy (ATP, *pmf*) for the producing organism. The amount of biologically useful energy harvested from the available Gibbs energy ($\Delta_r G$), however, depends on the metabolic pathways employed. These pathways can be very diverse, but they all have one thing in common: the produced acid (anions and H^+) must be exported. Zelle *et al.* recently showed for malic acid production in *S.cerevisiae* that the transport was crucial [10]. Therefore, part of the ATP or *pmf* produced in the network is needed for acid export, diminishing the ATP available for growth and/or maintenance. This export is common to all acids and, therefore, it is useful to consider it separately from metabolic pathway considerations. Metabolism occurs inside the cells where *p*H is considered to be constant at around $pH_i = 7$ which shows (Fig. 21.2) that the pathway produces a divalent (−2 charge) dicarboxylate anion and $2H^+$ ions. The anion and H^+ ions need to be exported from the cytosol by transport mechanisms. To be able to properly calculate the extent of transport using thermodynamic calculations, we first need to define the intracellular and extracellular conditions:

- The extracellular conditions cover a *p*H range between 1 and 8 outside of the cell (pH_o). For succinic acid (Fig. 21.2a), the total dissolved organic acid concentration decreases from 1 mol/Lat high pH_o to about 0.64 mol/L at low pH_o due to the presence of solid acid. For fumaric acid (Fig. 21.2b), the total dissolved organic acid concentration decreases from 1 mol/L at

high pH_o to only 0.047 mol/L at low pH_o due to the low solubility of the undissociated fumaric acid (Fig. 21.1).

- The intracellular conditions are characterized by neutral intracellular pH_i (=7). In addition, the intracellular concentration of dicarboxylic acid is needed. Recent measurements with *Escherichia coli* [11] or *Saccharomyces cerevisiae* [12] show concentrations on the order 10^{-3} mol/L.
- A very important aspect for the transport of charged molecular species is that the intracellular space has an electrical potential, ψ. Its value depends on pH_o (extracellular *p*H) in such a way that the so-called proton motive force (*pmf*) remains constant (homeostasis) when the pH_o changes. This *pmf*-homeostasis concept leads to the following relation between ψ (in Volts) and pH_o:

$$\psi = - \text{ pmf } - \frac{2.303RT}{F} (pH_o - pH_i) \tag{21.4}$$

In this relation, R and F are the gas constant (8.314×10^{-3} kJ mol^{-1} K^{-1}) and Faraday's constant (96.5 kJ Volt^{-1} e-mol^{-1}) and if $T = 298$K, then a value of 0.059 V is obtained for 2.303 RT/F. A typical value for the proton motive force is $pmf = 0.15$ V.

Equation 21.4 shows that the electrical potential inside is negative ($\psi = -0.15$V) when $pH_o = pH_i$, whereas at a lower extracellular *p*H (e.g., $pH_o = 3$), the potential becomes positive ($\psi = +0.084$ V).

The above defined situation around the membrane shows that we need a mechanism which can transport the dicarboxylic acid from a low inside total acid concentration of 0.001 mol/L to a much higher outside total acid concentration of 0.047 mol/L (fumarate) and 0.64 mol/L (succinate). Usually active transport is required where H^+ is involved in the export of one of the three acid-species (undissociated, A^- or A^{2-}). At $pH_i = 7$ and an intracellular total dicarboxylic acid concentration of 10^{-3} mol/L, one can calculate from the dissociation equilibria that the concentration of undissociated acid is 6.78×10^{-8} mol/L (succinic) and 4.90×10^{-10} mol/L (fumaric acid), while the concentration of mono-dissociated acid equals 4.18×10^{-5} mol/L and 1.23×10^{-7} mol/L, respectively. Apparently nearly all the intracellular acid is present as dicarboxylate, while the other species are, in general, present at less than μmol/L (10^{-6} mol/L) level. Therefore, we assume that the anion (2–) species is exported, together with export of n H^+ ions per mole of A^{2-} where n is an integer ($n = 0, \pm1, \pm2, \pm3, \ldots$). The anion export process can now be written as:

$$A^{2-}_{in} + nH^+_{in} \rightarrow A^{2-}_{out} + nH^+_{out} \tag{21.5}$$

which shows that the transport protein exports the anion (A^{2-}) together with n protons. The parameter n has different values for the different possible transport mechanisms:

for uniport $n = 0$; for symport and double symport $n = 1$ and 2; and for antiport and double antiport $n = -1$ and -2.

Using standard thermodynamics and recognizing that there is an intracellular electric potential (ψ), $\Delta_r G$ for the transport process of Equation 21.5 can be defined as:

$$\Delta_r G = 2.303\, RT \log\, (A_o^{2-} / A_i^{2-}) - 2.303nRT(p\mathrm{H_o} - p\mathrm{H_i}) - (n-2)\, F\psi \qquad (21.6)$$

where $A_{out}^{2-} = A_o^{2-}$ and $A_{in}^{2-} = A_i^{2-}$. We have already seen that the electrical potential, ψ, depends on the extracellular pH, $p\mathrm{H_o}$ (Eq. 21.4), and the *pmf*. Elimination of ψ leads to an expression for the achievable equilibrium out/in ratio of anions, A_o^{2-} / A_i^{2-}, ($\Delta_r G = 0$):

$$\log(A_o^{2-} / A_i^{2-})^{eq} = 2(p\mathrm{H_o} - p\mathrm{H}_i) + \frac{(n-2)(-pmf)F}{2.303RT} \qquad (21.7)$$

In this equation, *pmf* is (proton motive force) in Volts which is considered to have a typical value of +0.15 V and $2.303RT/F = 0.059$ V at 25 ºC. Equation 21.7 gives the equilibrium out/in ratio of the (–2) anion. In practice, one measures the total acid ratio. The equilibrium relation between the (–2) anion acid ratio (Eq. 21.7) and the total acid ratio, $(A_o/A_i)^{eq}$, follows from the dissociation relations (see also Eq. 21.3f and 21.3g):

$$\left(\frac{A_o}{A_i}\right)^{eq} = \left(\frac{10^{pK_1+pK_2-2p\mathrm{H}_o} + 10^{pK_2-p\mathrm{H}_o} + 1}{10^{pK_1+pK_2-2p\mathrm{H}_i} + 10^{pK_2-p\mathrm{H}_i} + 1}\right)\left(\frac{A_o^{2-}}{A_i^{2-}}\right)^{eq} \qquad (21.8)$$

Elimination of the (–2) anion acid ratio from Equations 21.7 and 21.8 gives the equilibrium out/in ratio of total acid A_o/A_i. The equilibrium total acid ratio, assuming a constant $p\mathrm{H_i}$ and *pmf*, only depends on $p\mathrm{H_o}$, $pK_{1,2}$ of the acid, and the number of protons (n) involved in the transporter mechanism ($n = 1,2$ for symport of 1 or 2 protons, $n = 0$ for uniport, $n = -1, -2$ for antiport of 1 or 2 protons).

Figure 21.5 shows that the achievable A_o/A_i equilibrium ratio drops steeply with decreasing $p\mathrm{H_o}$ until pH = 3 and increases steeply with decreasing n (from symport (n = 1) to uniport (n = 0) to antiport (n = –1)). Using Figure 21.5, we can select for each acid the thermodynamic feasible transport mechanism (number of protons involved) which yields the required A_o/A_i ratio at two relevant pH values (7 and 3). For *succinic acid* (Fig. 21.5a) the total acid concentration outside decreases from 1 mol/L (pH = 7) to 0.64 mol/L (pH = 3). Assuming 1 mmol/L as the inside concentration, the required value for the A_o/A_i ratio, as a function of pH,

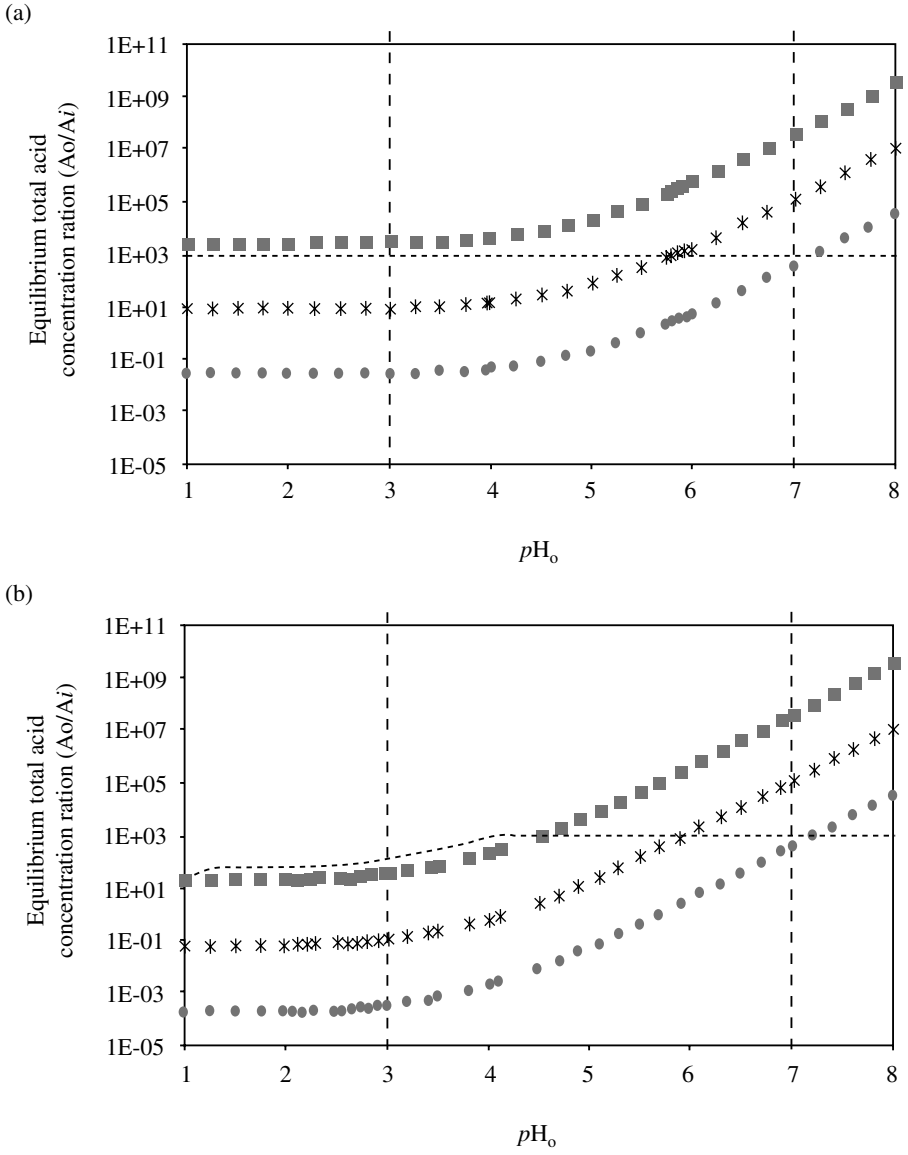

Figure 21.5 Out/in equilibrium total acid concentration ratio (A_o/A_i) as a function of $p\mathrm{H_o}$ for antiport ($n = -1$, squares), uniport ($n = 0$, stars), and symport ($n = 1$,circles): (a) succinic acid and (b) fumaric acid. The horizontal dashed line is the minimal required ratio ($A_i = 10^{-3}$mol/L).

is indicated (horizontal dashed lines) in Figure 21.5. To achieve sufficient driving force, the transport mechanism should achieve a value for the A_o/A_i equilibrium ratio which lies above the dashed line (Fig. 21.5). According to Figure 21.5(a), at $p\mathrm{H} = 3$ a mechanism where 1 $\mathrm{H^+}$ is antiported ($n = -1$) is required, whereas at

pH = 7 symport (n = 1) is suitable. For *fumaric acid* (Fig. 21.5b) the total dissolved acid decreases strongly with decreasing pH due to the low solubility of the undissociated acid. At pH = 7, all acid is dissolved and A_o/A_i = 1000 is required, thus, symport (n = 1) becomes a thermodynamically feasible mechanism. At pH = 3, the required total acid ratio is about 100 (A_o/A_i = 100), showing that antiport of 1 H^+, assuming $A_i = 3 \times 10^{-3}$ mol/L, is sufficient. We can conclude, concerning thermodynamically feasible transport mechanisms, that, for both acids, at pH = 7 a symport (n = 1) mechanism or at pH = 3 an antiport (n = −1) mechanism is required to achieve sufficient driving force for export. This conclusion becomes even stronger when A_{in}^{2-} is increased from 1 mmol/L to 10 mmol/L, showing that the choice of the A_{in}^{2-} value does not have a large effect on the result.

21.3.2 Metabolic energy required for dicarboxylic acid export

Previously, the thermodynamically feasible mechanisms for export of the dicarboxylic anion (A^{2-}) have been considered. However, the produced $2H^+$ must be exported as well. At pH = 7, symport is a feasible mechanism and the anion export takes care of 1 H^+, leaving another H^+ behind which still must be exported. At pH = 3, an antiport mechanism is needed for the anion export, meaning that $3H^+$ ions per anion still need to be exported. The H^+ export (which occurs against the proton motive force) requires a source of metabolic energy, which for the considered anaerobic conditions, is ATP. It is now important to distinguish prokaryotes from eukaryotes. In *prokaryotes*, such as the bacteria *E. coli*, H^+ is exported by a cell membrane bound H^+-ATPase enzyme at the expense of ATP. The stoichiometry (H^+ expelled per mole ATP consumed) is not certain, but can be set conservatively at 3 (see Appendix 21.A.3). This leads to a cost of 1/3 mole of ATP at pH = 7 and 1 mole of ATP at pH = 3 for the export of 1 mole of organic acid.

In *eukaryotes*, such as yeast or fungi which are considered as excellent production platforms at low pH, H^+ is expelled by an exporter which requires 1 ATP molecule per H^+ ion [13,14]. Thus, in eukaryotes at pH = 7 there is a 1 mole ATP cost and at pH = 3 a 3 mole ATP cost per mole of exported organic acid.

In summary, the export of dicarboxylic acid, using thermodynamically feasible transport mechanisms, requires the net export of 1 H^+ (pH = 7) and $3H^+$ (pH = 3) per mole of acid. For prokaryotes (e.g., *E. coli*), this translates per mole of organic acid at pH = 7 and 3 into a consumption of 1/3 and 1 mole of ATP and for eukaryotes (*S. cerevisiae*) into 1 and 3 moles of ATP. The massive ATP-consumption for acid export in eukaryotes is completely due to the inefficient and energetically wasteful means of H^+ transport over the cell membrane using H^+-ATPase (1 ATP only expels 1 H^+ compared to 3 H^+ in prokaryotes).

Clearly a genetic engineering target for eukaryotes is the introduction of a H^+-ATPase enzyme which maintains a H^+/ATP stoichiometry of 3 instead of 1 for H^+ export).

21.3.3 Converting Gibbs energy of the theoretical product reaction into ATP for growth

The previous thermodynamic analysis of the theoretical product reaction (Fig. 21.4) shows that for both acids at *p*H = 7 and *p*H = 3, there is a large Gibbs energy release (58 kJ for *p*H = 7 and 23 kJ for *p*H = 3 per mole of fumaric acid, as well as, 148 kJ for *p*H = 7 and 125 kJ for *p*H = 3 per mole of succinic acid). Only a part of this potentially available energy is converted into biological useful energy (ATP) depending on the metabolic network used for acid production. The biological energy (ATP) is needed for acid export, cell maintenance, and growth. A key issue is to analyze whether the product pathway, with the theoretical maximal product yield after taking the ATP need for export into account, provides a surplus of ATP to allow growth, because then an anaerobic process with only acid (fumaric or succinic) production, leading to the highest theoretical yield, can be performed. Even more important is the fact that evolutionary approaches can be used to improve the rate of product formation of engineered strains with this pathway, because faster production leads to faster growth due to ATP-based coupling between product formation and growth [15,16].

21.3.4 Fumaric acid

Figure 21.6(a) shows, for *p*H = 7, the network which anaerobically converts glucose to fumaric acid. First, we will discuss the energetically most favourable anaerobic network which uses the malic enzyme (as recently suggested [17]). An equivalent energy favourable alternative could be to introduce the reversible PEPCK [18]. Malic enzyme reductively carboxylates pyruvate (Pyr) to form malate without ATP consumption.The other energetically more expensive possibility is carboxylation of Pyr or phosphoenolpyruvate (PEP) to oxaloacetate (OAA), which requires 1 ATP per mole of C_4-acid. The pathway is redox neutral, because the glycolytic NADH (nicotinamide adenine dinucleotide [NAD] plus H^+) is consumed by the NADH coupled malic enzyme. The fumarate anion export (1 H^+ via symport) requires the additional export of 2 − 1 = 1 H^+/fumarate which is performed by a prokaryotic H^+-ATPase (stoichiometry of 3 H^+/ATP, see Appendix 21.A.3) demanding 1/3 of ATP per mole of acid. The net ATP production is then 2/3 of ATP per mole produced of fumaric acid, as indicated in the overall reaction (Fig. 21.6a). This amount is available for growth and maintenance and is equivalent to 2/3 × 45 kJ = 30 kJ of biological energy, which is about 50% of the potentially available energy (≈ 58 kJ at *p*H = 7, Fig. 4). The difference, 30 kJ per mole produced of acid, is needed to provide the thermodynamic driving force for each of the 13 reactions in the network.

Figure 21.6 shows the anaerobic fumarate network for *p*H = 7 or 3 (the only difference between Fig. 6(b) and 6(c) is the used H^+-ATPase). This difference results in a required export of $3H^+$ per mole of acid which consumes 1 ATP by the prokaryotic cell-membrane-bound H^+-ATPase. The end result is that the pathway produces

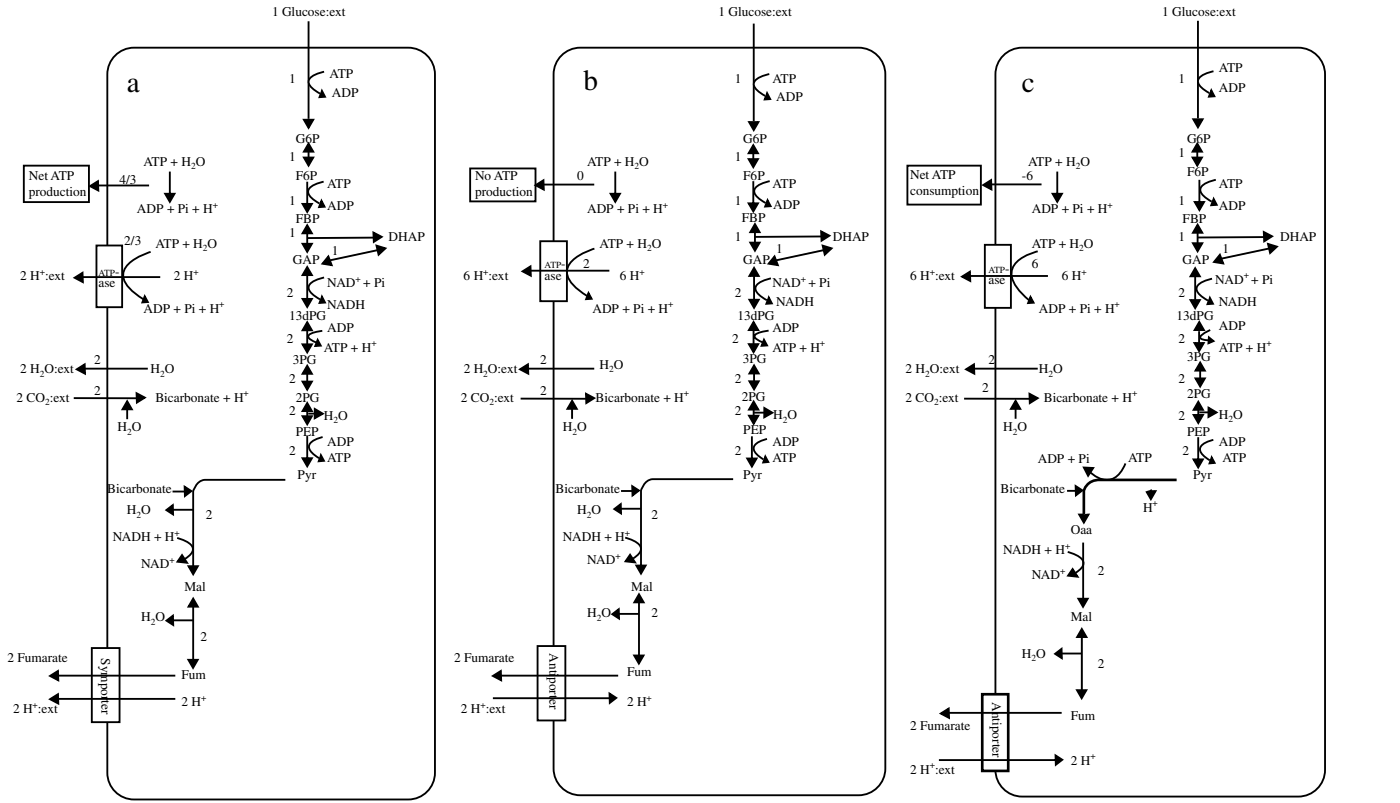

Figure 21.6 ATP analysis for the anaerobic fumarate network: (a) $p\mathrm{H} = 7$, symport for acid export, malic enzyme, $\mathrm{H^+/ATP} = 3$ (prokaryotic); (b) $p\mathrm{H} = 3$, antiport for acid export, malic enzyme, $\mathrm{H^+/ATP} = 3$ (prokaryotic); and (c) $p\mathrm{H} = 3$, antiport for acid export, pyruvate carboxylase, $\mathrm{H^+/ATP} = 1$ (eukaryotic).

no ATP, showing that the theoretically available Gibbs energy of 23 kJ (Fig. 21.4) does not allow any ATP synthesis. We could conclude that, in the fumarate product network, 20-30 kJ/mole of product is needed to assure a sufficient thermodynamic driving force for the 13 reactions. The ATP analysis shows that at *p*H = 7 one can perform an anaerobic fumaric acid process with 2 moles of fumaric acid/mole of glucose as a catabolic reaction with a 2/3 ATP yield. However, at *p*H = 3 this is not possible.

The above anaerobic network at *p*H = 3 (Fig. 21.6b) uses rather optimistic choices, such as the use of malic enzyme and a H^+/ATPase stoichiometry of 3. Other more realistic choices at this low *p*H are:

- a eukaryotic H^+/ATPase with H^+/ATP = 1, because at *p*H = 3 eukaryotes (yeast, fungi) are known to be much more acid tolerant than prokaryotes; and
- use of an ATP-consuming reaction for the carboxylation of C_3 (Pyr or PEP) to C_4 (oxaloacetic acid), e.g., pyruvate carboxylase.

Figure 6(c) then indicates at *p*H = 3 that a *consumption* of 3 ATP per mole of secreted fumaric acid is required, which then raises the question of how to generate the ATP.

Under anaerobic conditions, ATP production requires the production of ethanol or organic acids which is economically highly undesirable, because the fumaric acid yield decreases steeply and one obtains a product mixture (acids, alcohols) which renders DSP less efficient. The *aerobic* production of ATP is far more favourable, because there are no additional products (except CO_2 and H_2O) and the extra consumption of glucose to produce ATP is about 5-10 times less than under anaerobic conditions.

The aerobic fumarate production (Fig. 21.7) requires an excess production of NADH, which is converted to ATP in the electron transport chain (ETC). Therefore, in addition to the reductive (NADH consuming) fumarate pathway (Fig. 21.6), one needs an oxidative (NADH producing) fumarate pathway. This oxidative pathway involves the conversion of pyruvate to acetyl-CoA which is then followed by the glyoxylate pathway via succinate to fumarate.

Figure 21.7 shows the aerobic fumarate network at *p*H 3 which consists of a reductive branch, the oxidative glyoxylate branch, and the ETC (electron transport chain). The ETC exploits NADH/$FADH_2$ from the oxidative branch and the extracellular electron acceptor O_2 to produce the required ATP by oxidative phosphorylation. Furthermore, at *p*H = 3, fumarate is exported from the cell by H^+ antiport and the cytosolic H^+ is exported out of the cell via a eukaryotic H^+-ATPase (H^+/ATP = 1).

For a eukaryotic organism (yeast), the produced NADH and $FADH_2$ are consumed by the ETC then, it is assumed that, 1 NADH gives 1.25 ATP and 1 $FADH_2$ gives 0.75 ATP [19].

The aerobic fumarate network (combined reductive/oxidative path) with balanced ATP and redox then shows the following stoichiometry:

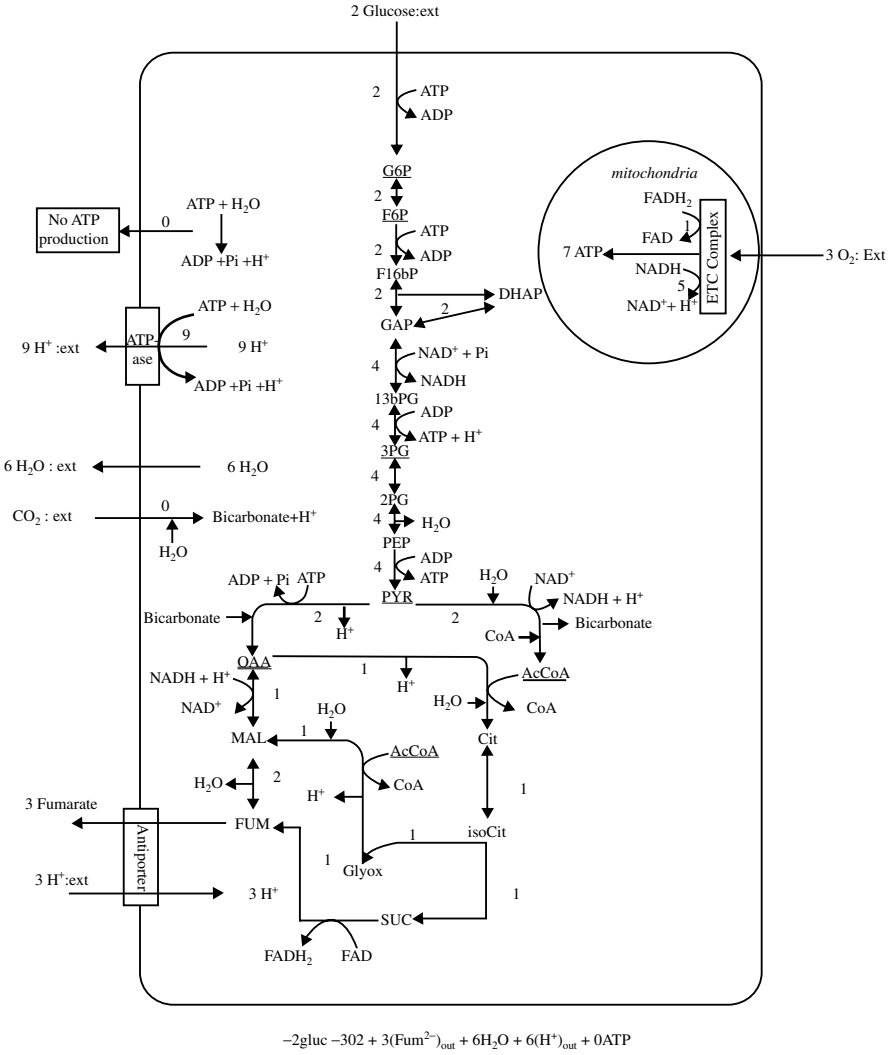

Figure 21.7 Network and stoichiometry for aerobic fumarate production at $p\mathrm{H} = 3$. Aerobic combined reductive and oxidative (glyoxylate) fumarate pathway with the electron transport chain (ETC) (NADH gives 1.25 ATP, $FADH_2$ gives 0.75 ATP), eukaryotic H^+-ATPase (H^+/ATP = 1), and ATP-requiring carboxylation of pyruvate. There is no growth and no net ATP production.

$$-2\text{glucose} - 3O_2 + 3(\text{Fum}^{2-})_{\text{out}} + 6H_2O + 6(H^+)_{\text{out}} \tag{21.9}$$

leading to a theoretical stoichiometry of 1.5 moles of fumarate/mole of glucose. This network requires that the α-ketoglutarate dehydrogenase enzyme is knocked out from the tricarboxylic acid (TCA) or Krebs cycle or, at least, down-regulated before entering the production phase.

21.3.5 Succinic acid

The black-box-thermodynamic result for succinic acid (Fig. 21.4) shows that there is much more Gibbs energy available than for the case of fumaric acid.

The anaerobic metabolic network for succinic acid is a combination of a reductive (which requires a net input of NADH for the reduction of fumarate to succinate) and an oxidative route which converts pyruvate to succinate and NADH via glyoxylate [20].

The NADH-balancing of the oxidative and reductive route leads back to the previous theoretical black-box reaction which has a yield of 24/14 moles of succinic acid per mole of glucose. The key question is now about ATP aspects of the succinate production which has received surprisingly little attention up to the present time.

For the reductive path, one usually makes traditional choices, such as the need of ATP for carboxylation of pyruvate to form oxaloacetate and for a cytosolic dehydrogenase to reduce fumarate to succinate. The overall *reductive reaction* is:

$$-1\text{glucose} - 2\text{NADH} - 2\text{CO}_2 + 2(\text{Succ})_{\text{in}} + 4(\text{H}^+)_{\text{in}} \tag{21.10}$$

And for the *oxidative (glyoxylate) pathway* it follows:

$$-1\text{glucose} + 1(\text{Succ})_{\text{in}} + (2\text{H}^+)_{\text{in}} + 2\text{ATP} + 5\text{NADH} + 2\text{CO}_2 \tag{21.11}$$

then multiplication of first reaction (Eq. 21.10) by 5, the second (Eq. 21.11) by 2, and then adding them together yields the black-box reaction, but in addition the ATP stoichiometry is obtained for production of 12 succinic acid:

$$-7\text{glucose} - 6\text{CO}_2 + 12(\text{Succ}^{2-})_{\text{in}} + 24(\text{H}^+)_{\text{in}} + 4\text{ATP} \tag{21.12}$$

However, for a full ATP-balance we need to take into the account the energy required for acid export.

At *p*H = 7, 1 H^+-symport represents a thermodynamically feasible export mechanism, leaving 12 H^+ ions to be exported by H^+-ATPase (in reality the export of 12 Suc^{2-} and 12 H^+). Assuming a prokaryotic ATPase, which exports 3 H^+ at the cost of 1 ATP, then, for the export of 12 H^+ ions, 12/3 = 4 ATP molecules would be needed, leading to a zero net-balance of ATP per mole of secreted succinic acid. Hence, anaerobic succinate production at *p*H 7 using this traditional network *does not provide ATP*. This means that ATP must be produced using traditional pathways (resulting in ethanol and/or acetate secretion). Indeed, all published reports of prokaryotic systems producing succinate at *p*H = 7 mention the additional presence of these by-products [21-30]. The production of unwanted by-products, according to our analysis, is completely due to the generation of ATP which is needed to supply energy for cell maintenance and growth.

At pH = 3, the H^+-antiport transport mechanism leads to 36 moles of H^+ which must be exported. At low pH, it is generally accepted that eukaryotes are more acid-tolerant than prokaryotes and, therefore, it is useful to assume the presence of a eukaryotic H^+-ATPase (H^+/ATP = 1) which consumes 36 moles of ATP to export 12 succinate molecules, meaning that 36 − 4 = 32 additional moles of ATP must be generated.

We observe now a tremendous discrepancy between the potentially available (Fig. 21.4) Gibbs energy (which would allow the maximum production of about 2 ATP (pH = 3) or 3 ATP (pH = 7) per mole of secreted succinate) and the net ATP balance of the conventional network (no ATP at pH = 7 and a consumption of 32/12 moles of ATP per mole of succinic acidat pH = 3). Thus, there are major losses of Gibbs energy in the network. Global inspection of reaction thermodynamics points to 3 reactions: the eukaryotic H^+-ATPase, the ATP requiring carboxylation of pyruvate, and the fumarate reduction to succinate (Appendix 21.A.3).

Figure 21.8(a) and 21.8(b) shows (for pH = 3 and 7) alternative networks where the cytosolic fumarate reduction with NADH is replaced by a membrane bound reduction exploiting a menaquinone based electron transport chain which exports H^+ using the large Gibbs energy release of the reduction reaction.

Appendix 21.A.3 shows that we can expect an export of $3H^+$ per reduced fumarate. This proton export partly replaces the use of the highly unfavourable eukaryotic H^+-ATPase and, therefore, is of crucial importance. In addition, it is assumed that the excess H^+ exported outside of the cell is then imported back into the cell by a reversible H^+ consuming ATPase (prokaryote with 3 H^+/ATP) in order to make ATP.

The modified fumarate reduction produces succinate, but ATP-production is also possible under anaerobic conditions at pH = 3 with with 2/12 moles of ATP and at pH = 7 with 10/12 moles of ATP per mole of secreted acid. These networks then conserve per mole of succinic acid an amount of energy (in terms of ATP) in the range of 8 kJ (pH = 3, 2/12 × 45 kJ) to 38 kJ (pH = 7, 10/12 × 45 kJ) which is only 6% to 25% of the available energy (Fig. 21.4). Of course, the ATP yield will increase when the ATP-requiring carboxylation (pyruvate carboxylase) is replaced by another enzyme, e.g., the one for malic acid [17]. This change will increase the produced ATP per succinate ratio to 12/12 (pH = 3) and 20/12 (pH = 7), as well as the Gibbs energy efficiency to 36% to 51%. The Gibbs energy loss in this much more complex network with 20 reactions is then around 75 kJ/mole of succinate.

21.3.6 Acid back diffusion

A final energy aspect at low pH is that the secreted undissociated, organic acid diffuses back [31] at a rate determined by the extracellular concentration of the undissociated acid and the membrane permeability constant for the acid (which depends on the acid solubility and diffusivity in the membrane). For fumaric acid at low pH, the concentration is constant (solubility limit) and, therefore (assuming constant permeability), the back-diffusion flux is constant (q^{bd}_{Fum} = 20.0 mmol acid h^{-1} C-mol X^{-1}; based on

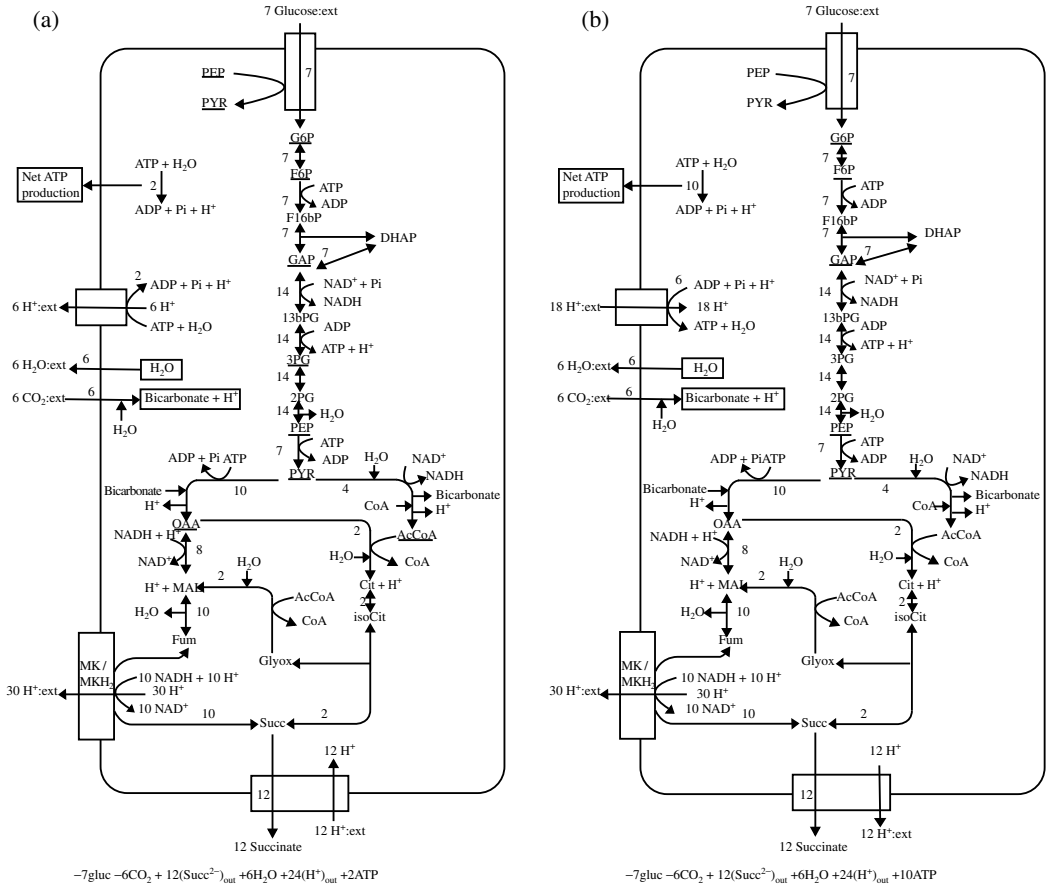

Figure 21.8 Succinic acid production network using a membrane bound, H^+ translocating, menaquinone-mediated fumarate reduction (to succinate) and ATP-generation from the proton motive force (*pmf*) (prokaryotic 3 H^+ uptake per ATP) at: (a) pH = 3 (using H^+ antiport for A^{2-} export) and (b) pH = 7 (using H^+ symport for A^{2-} export).

measured data). Figure 21.9(a) shows the relationship between specific fumarate productivity (q_{Fum}) and the glucose and O_2 consumption (per mole of fumarate) using the network of Figure 21.7 which now includes acid back-diffusion (see Appendix 21.A.4 for the detailed calculations). Obviously this back-diffused acid must be exported out

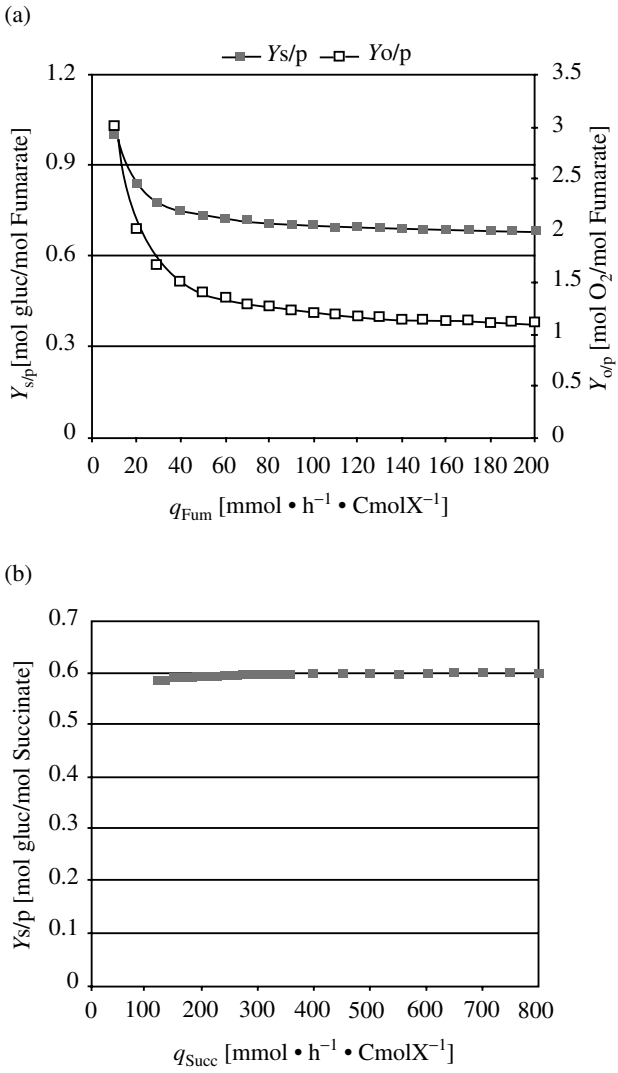

Figure 21.9 (a) Glucose and O_2 required per mole of fumarate as a function of the specific fumarate production rate (q_{Fum}), assuming the network of Figure 21.7 and a fumarate back-diffusion rate of 20 mmol h^{-1} C-mol X^{-1}, (b) glucose required per mole of succinate as a function of the specific succinate production rate (q_{Succ}), assuming the network of Figure 21.8(a) and a succinate back-diffusion rate of 20 mmol h^{-1} C-mol X^{-1}.

of the cell again at high energy cost, where the consumption of glucose and O_2 per mole of fumarate increases with decreasing q_{Fum}.

Also, undissociated succinic acid can diffuse back, leading to aneed of additional ATP for the export of this acid (see Appendix 21.A.4). However, in contrast to the fumaric acid case, there is a decrease in the glucose consumed per mole of succinic acid, because less ATP is needed for growth. Figure 21.9(b) shows that, as the specific succinate productivity (q_{Succ}) decreases, the glucose consumed per mole of succinate also decreases, albeit slightly.

21.4 GENETIC ENGINEERING OF TARGET SYSTEMS BASED UPON THERMODYNAMIC ANALYSIS RESULTS

For real networks the harvested ATP-energy must be less then the available Gibbs energy. Therefore, it is of interest to analyze the Gibbs energy losses which occur per reaction. This thermodynamic approach is highly useful for the development of genetically engineered target systems which use energy from ATP more efficiently and have a higher product yield.

A clear genetic-engineering target in eukaryotes is the introduction of a cell membrane bound H^+-ATPase, with a H^+/ATP stoichiometry of 3 instead of 1 (as in the ABC transporter), for H^+ export.

For succinic acid, an important target is to couple fumarate reduction to proton motive force (*pmf*) generation. For both acids, replacing pyruvate carboxylase with the malic enzyme for anaplerosis [17,18] will increase the ATP production and, thus, decrease the ATP cost. With all of these modifications, around 30% to 50% of the available Gibbs free energy can be converted to ATP.

21.5 CONCLUSION

The microbial production of the dicarboxylic acids, succinic and fumaric, from glucose at low *p*H was analyzed using a thermodynamic framework. First the black box (maximal) theoretical product reaction was studied with respect to the stoichiometry of the individual dicarboxylic acid species and the Gibbs energy formation of these reactions were obtained as a function of *p*H (in the range of 1-8). The results were very promising with very negative values for $\Delta_r G$ (kJ/mol acid) for both dicarboxylic acids. However, at lower *p*H usually less Gibbs energy is released. The thermodynamically feasible mechanisms for carboxylic acid and H^+ export were discussed, including their energy need, and candidate metabolic networks were analyzed with respect to conversion of available Gibbs energy into ATP.

Here, we show that the analysis of energy aspects of both the product reaction and metabolic network, including transport, is vital, because it elucidates the strong and weak points of the network, in terms of energy efficiency, and directly suggests genetic-engineering targets to further improve the product yield, with the goal of achieving ultimately anaerobic production.

The energy analysis shows that it seems possible (after implementing the suggested genetic modifications) that succinic acid (at *p*H 3 and 7) and fumaric acid at *p*H 7 can be produced anaerobically as a sole catabolic product with a non-zero net ATP production. At low *p*H (3) fumaric acid production must be performed aerobically. Finally, the consequences for acid back-diffusion at low *p*H (3) are found to be different for both acids, leading to lower aerobic fumarate and higher anaerobic succinate yields.

21.A APPENDICES

21.A.1 Acid/alkali cost

The cheapest alkali is $CaCO_3$ which costs about € 270/ton (98% pure). A high purity is needed to produce an acid of high purity acid. The cheapest acid for downstream processing is sulphuric acid (H_2SO_4) which costs about € 90/ton (98% pure). For 1 mole of H^+, there is a cost of 0.0045 € and for 1 mole of OH^- 0.0135 €. The total acid/alkali cost per kg of dicarboxylic acid is then € 0.30/kg. In addition, there are costs for disposal of the produced $CaSO_4$.

21.A.2 Standard $\Delta_f G$ values

Table 21.A1 Standard Gibbs energy of formation ($\Delta_f G^o$) for various species at 25°C and zero ionic strength [32].

Compound	$\Delta_f G^o$ [kJ]
Glucose	–915.90
H_2Fum (s) [a]	–645.68
H_2Fum (aq)	–645.80
$HFum^-$(aq)	–628.14
Fum^{2-}(aq)	–601.87
H_2Succ (s) [a]	–747.75
H_2Succ (aq)	–746.64
$HSucc^-$(aq)	–722.62
$Succ^{2-}$(aq)	–690.44
H_2O	–237.19
H^+	0
CO_2(g)	–394.36

[a] $\Delta_f G^o$ values for the solid forms of fumaric and succinic acid are obtained by the knowledge that the solid and aqueous forms are in equilibrium when the concentration of the aqueous form reaches the solubility limit: $\Delta_f G^o_{H_2A(s)} = \Delta_f G^o_{H_2A(aq)} + RT\ln(c^*_{H_2A(aq)})$, $c^*_{H_2A(aq)}$ is the solubility (mol/L) of fumaric or succinic acid in water at 25 °C.

21.A.3 In vivo energy aspects of ATP, proton motive force, and fumarate reductase

ATP

ATP is synthesized according to ADP + $P_i \rightarrow$ATP. The Gibbs energy (kJ/mol ATP) of the reaction is $\Delta_r G^o = 30.9$ kJ/mol [33].

$$\Delta_r G_{ATP} = +30.9 + RT \ln\left(\frac{ATP}{ADP\ P_i}\right)$$

The indicated concentrations are given in Table 21.A2. The value of ATP/(ADP P_i) ≈ 100, leading to an *in-vivo* value (25 ºC) of $\Delta_r G_{ATP} \approx 45$ kJ/mol.

Table 21.A2 Concentrations of nucleotides and P_i for aerobic production in glucose limited *S. cerevisiae*and *E. coli* (mmol/L).

	S. cerevisiae	*E. coli*
ATP	2.9 ± 0.1[a]	3.36 ± 0.03[c]
ADP	0.71 ± 0.04[a]	1.30 ± 0.01[c]
P_i	43[b]	–

[a]taken from Canelas *et al.* [12]. Concentrations were converted to mmol/l using the factor 2.38 mLcell/gDW [34].
[b]taken from Wu *et al.* [35].
[c]taken from Taymaz-Nikerel *et al.* [11]. Concentrations were converted to mmol/l using the factor 1.77 mLcell/gDW[36].

Proton motive force (pmf)

A typical *pmf* value is 0.15 Volts which translates to, using Faraday's constant (96.5 kJ $Volt^{-1}e–mol^{-1}$), $0.15 \times 96.5 = 14.5$ kJ Gibbs energy needed to export 1 mole of H^+.

With the above result for $\Delta_r G_{ATP}$, it follows that $3H^+$ ions can be exported per 1 mole of ATP hydrolysis by the reversible H^+-ATPase.

Membrane bound fumarate reductase in succinate production

Membrane bound fumarate reductase reduces fumarate to succinate using menaquinone MKH_2 as an electron donor. This reaction is of particular importance, because it is a potential rich source of biological energy. Harvesting this energy (as *pmf*) requires that the reduction occurs by a set of membrane coupled proteins

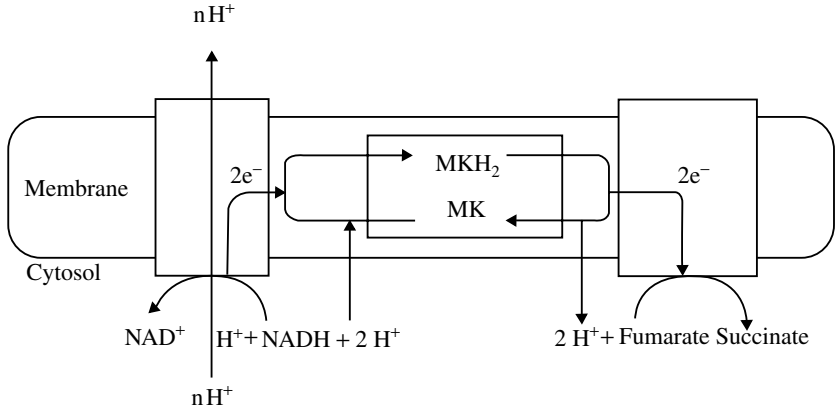

Figure 21.A1 Schematic of the fumarate respiratory chain.

which form an electron transport chain (ETC) with MKH_2 as the intermediate electron donor and fumarate as the electron acceptor. This chain expels H^+ and generates *pmf*.

Figure 21.A1 shows the proposed mechanism which consists of two reactions:

- The first reaction reduces fumarate to succinate using reduced menaquinone as the electron donor. The equilibrium constant follows from the respective standard redox potentials, K_{eq} = 6895 (25 ºC). Measurements of fumarate and succinate show a typical succinate/fumarate concentration ratio of about 0.10 [11], leading to a maximum ratio of $MK/MKH_2 \approx 690$ showing that nearly all of the menaquinone is oxidized.
- The second reaction regenerates MKH_2 from MK using NADH and expels H^+:

$$NADH + MK + mH^+_{in} \rightarrow NAD^+ + MKH_2 + mH^+_{out}$$

First we consider $\Delta_r G$ in the absence of H^+ expulsion. The $\Delta_r G^o$ of this reaction is –47.5 kJ/mol (using $\Delta E^1_o = -0.32$ V for $NADH/NAD^+$ [37] and $\Delta E^1_o = -0.074$ V for MKH_2/MK [38]). Under *in vivo* conditions, $NADH/NAD^+ \approx 0.01$ in *S. cerevisiae* [39] and 0.02 in *E.coli* [11] which gives an average $NAD^+/NADH \approx 75$. Also as already mentioned above, $MK/MKH_2 \approx 690$.

The *in-vivo* Gibbs energy of reaction is:

$$\Delta_r G = -47.5 + RT \ln\left(\frac{75}{690}\right) = -52 \text{ kJ/mol}$$

This amount of Gibbs energy is available to expel protons. For export of 1 mole of H^+, given the *pmf* value of 0.15 Volts, one needs 14.5 kJ, hence, the maximum possible stoichiometry is 3 moles of H^+ expulsion per mole of NADH (which consumes $3 \times 14.5 = 43.5$ kJ from the available 52 kJ). The conclusion is that membrane coupled fumarate reductase using NADH can lead to the export of 3 moles of H^+.

21.A.4 Effect of acid back-diffusion on the product yield of dicarboxylic acid

Fumaric acid

Fumaric acid, at *p*H = 3, shows back-diffusion (at a rate of 20 mmol h^{-1} C-mol X^{-1}) which is constant due to the solubility limit (47 mmol/L) of undissociated fumaric acid. The acid is exported by an H^+-antiporter and, in addition, 3 H^+ per mole of fumaric acid needs to be exported. Assuming a eukaryotic H^+-ATPase (H^+/ATP = 1 mol/mol), acid back-diffusion and its export create a futile cycle which requires $3 \times 20 = 60$ mmol ATP h^{-1} C-mol X^{-1}.

The *catabolic reaction* of glucose (P/NADH = 1.25 and P/FADH = 0.75) leads to 4 (substrate phosphorylation) plus $10 \times 1.25 + 2 \times 0.75 = 14$ (oxidative phosphorylation) = 18 moles of ATP, hence we can write:

$$-1\text{glucose} - 6O_2 + 6CO_2 + 6H_2O + 18\text{ATP}$$

Figure 21.7 shows the stoichiometry of the *fumaric acid formation reaction*:

$$-2\text{glucose} - 3O_2 + 3(\text{Fum}^{2-})_{\text{out}} + 6H_2O + 6(H^+)_{\text{out}}$$

which gives for the substrate and O_2 consumption as a function of q_{Fum}:

$$q_S = -\frac{60}{18} - \frac{2}{3}q_{\text{Fum}}$$

$$q_O = -\frac{6 \times 60}{18} - \frac{3}{3}q_{\text{Fum}}$$

The glucose and O_2 consumption per mole of fumarate then becomes:

$$\frac{\text{mol glucose}}{\text{mol fumarate}} = \frac{60/18 + (2/3)q_{\text{Fum}}}{q_{\text{Fum}}}$$

$$\frac{\text{mol } O_2}{\text{mol fumarate}} = \frac{6 \times \frac{60}{18} + (3/3) q_{\text{Fum}}}{q_{\text{Fum}}}$$

These yields are shown in Figure 21.9(a). At high productivity, one achieves asymptotically the lowest consumption of 0.667 $\frac{\text{mol glucose}}{\text{mol fumarate}}$ and 1 $\frac{\text{mol } O_2}{\text{mol fumarate}}$.

Succinic acid

It is expected that succinic acid also back diffuses at pH = 3. The back-diffusion rate is assumed to be the same as in the fumaric acid case (constant at 20 mmol h^{-1} C-mol X^{-1}), because succinic acid has a much higher solubility, but, on the other hand, has a lower permeability.

The acid is exported by an H^+-antiporter and, in addition, 2 H^+ per mole of succinic acid needs to be exported (in total $3H^+$). A prokaryotic H^+-ATPase ($3H^+$/ATP), acid back-diffusion, and its export require 1 ATP per mole of succinic acid. The *back diffusion reaction* with a rate q_{bd} is:

$$-(\text{Succ}^{2-})_{\text{in}} - 1\ \text{ATP} + (\text{Succ}^{2-})_{\text{out}}$$

the *anaerobic production reaction* at pH = 3 (as in Fig. 21.8a) with a rate q_{Succ} is:

$$-7\ \text{glucose} - 2\ CO_2 + 12\ (\text{Succ}^{2-})_{\text{out}} + H_2O + (H^+)_{\text{out}} + 2\ \text{ATP}$$

The *growth reaction* with a rate μ is:

$$-1/6\ \text{glucose} - 1.5\ \text{ATP} - \ldots + 1\ \text{Biomass} + \ldots$$

the ATP balance is:

$$\frac{2}{12} q_{\text{Succ}} - 1.5\mu - 1 q_{\text{bd}} = 0\text{, which gives } q_{\text{Succ}} = 9\mu + 6 q_{\text{bd}}$$

and the substrate rate will be:

$$-q_S = \frac{7}{12} q_{\text{Succ}} + \frac{1}{6}\mu$$

Because 1/6 ATP is required per mole of secreted succinate, the minimum net succinate production rate would be 20/(1/6) = 120 mmol h^{-1} • Cmol X^{-1}, as shown in Figure 21.9(b).

LIST OF SYMBOLS

A	acid
c^*	solubility [mol/L]
ΔE^1_o	difference in redox potential [Volts]
F	Faradays constant (96.5 kJ $Volt^{-1}$e-mol^{-1})
$\Delta_r G$	Gibbs free energy of reaction [kJ/mol]
$\Delta_f G^o$	standard Gibbs free energy of formation [kJ/mol]
K_{eq}	equilibrium constant
n	number of protons
P_i	inorganic phosphate
pK	dissociation equilibrium constant
pmf	proton motive force [Volts]
q	biomass specific rate [mmol h^{-1} C-mol X^{-1}]
R	gas constant (8.314×10^{-3}kJ mol^{-1} K^{-1})
T	temperature
γ	yield
μ	specific growth rate
ψ	electrical potential [Volts]

Subscripts/Superscripts

bd	back diffusion
eq	equilibrium
Fum, F	fumarate
i	in
o	out
P	product
S	substrate
Succ	succinate

ABBREVIATIONS

13bPG, 1-3biphosphoglycerate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; AcCoA, Acetyl Coenzyme A; ADP, adenosine-5-diphosphate; AMP, adenosine-5-monophosphate; ATP, adenosine-5-triphosphate; Cit, citrate; DHAP, dihydroxiacetone phosphate; DSP, downstream processing; ETC, electron transport chain; F16bP, fructose-1,6-bisphosphate; F6P, fructose-6-phospate; Fum, fumarate; G6P, glucose-6-phosphate; GAP, glycerol-3-phosphate; GLyox, glyoxylate; MAL, malate; MK(H2), menaquinone (reduced form); NAD(H), nicotinamide adenine dinucleotide (reduced

form); NADP(H), nicotinamide adenine dinucleotide phosphate (reduced form); OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; TCA, tricarboxylic acid.

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