Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 176-182

AN ATP/2e$^-$ STOICHIOMETRY OF 1½ IS THERMODYNAMICALLY POSSIBLE FOR SITE 3 OF OXIDATIVE PHOSPHORYLATION

John J. Lemasters and Kenneth E. Fleishman

Laboratories for Cell Biology, Department of Anatomy, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Received November 18, 1986

Summary. Free energy changes for ATP synthesis (ΔG_P) and 2e$^-$-transfer across Site 3 (ΔG_R) were determined during oxidative phosphorylation by rat liver mitochondria. At static head, $-\Delta G_R/\Delta G_P$ ranged narrowly between 1.55 and 1.59 with five different respiratory substrates. Thus, an ATP/2e$^-$ of 1½ at Site 3 is thermodynamically possible with regards to overall reactants and products. Using nonequilibrium thermodynamics, phenomenological stoichiometries were close to 1½ for all substrates suggesting that ATP/2e$^-$ at Site 3 is, in fact, 1½. An ATP/2e$^-$ of 1½ can only be possible if H^+/O is 4 for cytochrome oxidase.

The terminal step of mitochondrial respiration is catalyzed by cytochrome c oxidase, the classical Site 3 of oxidative phosphorylation. In chemiosmotic theory, energy conservation during respiration is mediated by H^+ and/or charge translocation. Presently, H^+/O at Site 3 is held to be either 2 or 4 [1,2]. The assumption is made that oxygen is reduced to water on the matrix side of the membrane and that $2H^+/O$ are consumed by this scalar reaction. Adding these scalar H^+, the net or charge stoichiometry at Site 3 becomes 4 or 6 for the two models under consideration.

ATP/2e$^-$ at any site is the quotient of the charge stoichiometry and the sum of the H^+ stoichiometries of the H^+-translocating ATPase and the ATP, ADP and Pi transport system. ATP exchange for ADP and Pi is coupled to the movement of $1H^+$ across the membrane, and the H^+ stoichiometry of the ATPase is held to be either 2 or 3 [see ref. 3 for discussion]. Thus, either 3 or $4H^+$ are required to synthesize and transport ATP from extramitochondrial reactants. Since neither the charge stoichiometry of cytochrome oxidase nor the H^+ stoichiometry of mitochondrial ATPase is established, there are four possibilities for ATP/2e$^-$ at Site 3: if charge/O is 4, then ATP/2e$^-$ is 1 or $1\frac{1}{3}$; if charge/O is 6, then ATP/2e$^-$ is 1½ or 2.

On thermodynamic grounds, ATP/2e$^-$ cannot exceed $-\Delta G_R/\Delta G_P$ where ΔG_R and ΔG_P are free energy changes of 2e$^-$-transfer across Site 3 and ATP synthesis from

0006-291X/87 $1.50

ADP and Pi, respectively. Here, in order to determine which ATP/$2e^-$ stoichiometries are thermodynamically possible at Site 3, ΔG_R and ΔG_P were determined during static head oxidative phosphorylation by rat liver mitochondria under a variety of reaction conditions.

METHODS

Rat liver mitochondria were incubated at 3 mg of protein/ml in 150 mM sucrose, 0.5 mM EDTA or 5 mM $MgCl_2$, 5 or 10 mM ATP, 100 or 125 μM ADP, 1 mM KPi and 25 mM K-HEPES buffer, pH 7.4, 23°C. Respiratory substrate was 5 mM succinate plus 2 μM rotenone, 5 mM L-glutamate plus 5 mM L-malate, 5 mM L-pyruvate plus 5 mM L-malate, 10 mM DL-3-hydroxybutyrate, or 5 mM 2-oxoglutarate. After 4 minutes, samples were taken for determination of ATP, ADP and Pi. Parallel incubations were monitored for pH and cytochrome $\underline{c}+\underline{c}_1$ reduction at the wavelength pair, 550-540 nm [4].

Mitochondria generate high ATP/ADP ratios at static head. Small amounts of ADP in the matrix or from ATP hydrolysis during quenching and extraction can therefore cause disproportionate changes in the apparent ATP/ADP ratio and extramitochondrial ΔG_P. To minimize these extraneous sources of ADP, suspensions were filtered, and the filtrates subjected to an organic quenching and extraction procedure [5]. Isocratic, reverse phase high pressure liquid chromatography was employed to determine ATP and ADP [6]. Inorganic phosphate was determined by a zinc molybdate procedure correcting for hydrolysis of organic phosphates during the assay [7].

ΔG_P was calculated as previously described correcting for pH, temperature, ionic strength and Mg^{++} concentration [8]. Rosing and Slater's [9] value for $\Delta G_P°'$ was used throughout. ΔE, the oxidation-reduction potential across Site 3, was calculated from the partial pressure of oxygen and the reduced to oxidized ratio of cytochrome $\underline{c}$ using values for Em at pH 7 of 230 mV for cytochrome $\underline{c}$ and 816 mV for water/oxygen [10, 11]. ΔE was corrected for actual pH by -59 mV/pH unit. ΔG_R was calculated from ΔE where $\Delta G_R = -2F\Delta E$. The degree of coupling, q, was estimated from the respiratory control ratio (RCR) and ΔG_P before and after ADP addition [4,8]. In general, each coupling site will have its own degree of coupling. However, since ΔG_R measured across Site 3 was essentially unchanged after ADP addition ($\Delta\Delta G_R$ < 1 kJ/mol), q specific for Site 3 will not be appreciably different from q for the overall reaction. The phenomenological stoichiometry, Z, was evaluated at static head where $Z = q(-\Delta G_R/\Delta G_P)$.

RESULTS AND DISCUSSION

For mitochondria oxidizing various substrates at static head, ΔG_P, ΔE and ΔG_R ranged between 62.1 and 65.3 kJ/mol, 507 and 526 mV, and -97.8 and -101.5 kJ/mol, respectively (Table I). Similar values were observed in the presence and absence of added Mg^{++}. Succinate supported the largest values for ΔG_P, ΔE and $-\Delta G_R$ whereas 3-hydroxybutyrate and 2-oxoglutarate produced the smallest values. As a result, $-\Delta G_R/\Delta G_P$ was nearly constant, ranging narrowly between 1.55 and 1.59. In Mg^{++}-free medium, $-\Delta G_R/\Delta G_P$ was 1.55 or 1.56 for all substrates. These ratios obtained with 5 different respiratory substrates in two different incubation mediums agree with a single earlier observation for rat liver mitochondria oxidizing 3-hydroxybutyrate/acetoacetate

TABLE I
Equilibrium thermodynamics of static head oxidative phosphorylation across Site 3

Substrate	ATP (mM)	ADP (mM)	Pi (mM)	pH	$\underline{c}^{+2}/c^{+3}$	O_2 (atm)	ΔG_P (kJ/mol)	ΔE (mV)	ΔG_R (kJ/mol)	$-\Delta G_R/\Delta G_P$
Succinate										
$+MgCl_2$	9.79	0.049	0.654	7.42	0.469	0.082	64.1	526	-101.5	1.58
$-MgCl_2$	4.90	0.019	0.829	7.37	0.394	0.095	65.3	526	-101.4	1.55
Glutamate plus malate										
$+MgCl_2$	9.79	0.042	0.790	7.42	0.278	0.126	64.1	515	-99.5	1.55
$-MgCl_2$	4.88	0.038	0.829	7.39	0.275	0.118	63.8	517	-99.7	1.56
Pyruvate plus malate										
$+MgCl_2$	9.79	0.063	0.705	7.42	0.263	0.139	63.3	515	-99.3	1.57
$-MgCl_2$	4.88	0.037	0.915	7.39	0.212	0.135	63.6	516	-98.6	1.55
3-Hydroxybutyrate										
$+MgCl_2$	9.78	0.060	0.875	7.42	0.196	0.134	62.9	507	-97.8	1.55
$-MgCl_2$	4.88	0.031	0.866	7.25	0.174	0.125	63.5	513	-99.1	1.56
2-Oxoglutarate										
$+MgCl_2$	9.78	0.072	1.01	7.42	0.233	0.141	62.1	511	-98.7	1.59
$-MgCl_2$	4.87	0.040	0.863	7.35	0.225	0.136	63.3	509	-98.3	1.55

Rat liver mitochondria were incubated with various substrates in the reaction medium described in METHODS. After 4 minutes, ATP, ADP, Pi, pH and O_2 were measured in the extramitochondrial phase, and the oxidation-reduction state of endogenous mitochondrial cytochrome $\underline{c}$ was determined. Other experimental details are as described in METHODS.

in Mg^{++}-free medium [4]. On thermodynamic grounds, ATP/$2e^-$ must be less than or equal to $-\Delta G_R/\Delta G_P$. Therefore, ATP/$2e^-$ stoichiometries of 1½, 1⅓ and 1 are all possible with regards to overall reactants and products across Site 3. An ATP/$2e^-$ ratio of 2, however, is clearly impermisible.

The theory of linear nonequilibrium thermodynamics provides a means for estimating the mechanistic ATP/$2e^-$ stoichiometry, n, from static head force ratios. Two parameters must be evaluated: the degree of coupling, q, and the phenomenological stoichiometry, Z. For linear reciprocal systems, n will lie between Z/q and qZ. For tightly coupled systems where q approaches 1, Z will approximate n [3,4].

For each experimental condition, q was evaluated from RCR and ΔG_P before and after respiratory stimulation with ADP. As a consequence of low Pi and high ATP in the reaction medium, RCR's were small, ranging between 2.1 and 3.9. At higher Pi

TABLE II
Nonequilibrium thermodynamics of static head oxidative phosphorylation across Site 3

Substrate	RCR	ΔG_P (kJ/mol)	q	Z
Succinate				
$+MgCl_2$	3.80	58.4	0.984	1.55
$-MgCl_2$	2.48	57.4	0.961	1.49
Glutamate plus malate				
$+MgCl_2$	3.92	58.0	0.984	1.53
$-MgCl_2$	2.61	57.4	0.970	1.51
Pyruvate plus malate				
$+MgCl_2$	3.40	58.2	0.983	1.54
$-MgCl_2$	2.14	57.1	0.958	1.48
3-Hydroxybutyrate				
$+MgCl_2$	3.12	57.6	0.981	1.52
$-MgCl_2$	2.21	56.6	0.958	1.49
2-Oxoglutarate				
$+MgCl_2$	3.72	57.2	0.986	1.57
$-MgCl_2$	2.64	57.0	0.971	1.51
		Average	0.974	1.52
		S.D.	0.011	0.03

Rat liver mitochondria were incubated at static head as in TABLE I. After 4 minutes, 500 μM ADP was added to determine RCR. After another minute, a sample was taken to determine ΔG_P. q was calculated with eq. 11 of ref. [8] and Z by the relation, $Z = -q(\Delta G_R/\Delta G_P)$, using static head values for $-\Delta G_R/\Delta G_P$ from TABLE I.

concentrations in the absence of ATP, RCR's were between 5 and 8 (data not shown). From these measurements, q averaged 0.974 and was slightly greater in Mg^{++}-containing than in Mg^{++}-free medium (Table II). Z calculated from q and $-\Delta G_R/\Delta G_P$ ranged from 1.48 to 1.57 with a mean of 1.52.

For each respiratory substrate, then, the results of the nonequilibrium thermodynamic analysis support an $ATP/2e^-$ stoichiometry of 1½ as predicted by a chemiosmotic scheme in which H^+ stoichiometries are 4 for cytochrome oxidase, 3 for the ATPase and 1 for the transport of ATP in exchange for ADP and Pi. It must be emphasized, however, that unlike equilibrium thermodynamics, the conclusions of nonequilibrium thermodynamics depend on the validity of the assumptions of linearity and reciprocity. Although linearity and reciprocity have been demonstrated in rat liver mitochondria

and submitochondrial particles [12,13], the generality of linearity and reciprocity for oxidative phosphorylation has not yet been established. Thus, the data presented here indicate that the ATP stoichiometry for Site 3 must be less than or equal to about 1.56, the average value for $-\Delta G_R/\Delta G_P$ at static head, but the agreement of Z with a particular model may simply be fortuitous. However, Z was virtually invariant for all conditions examined, and previous work has shown that nonequilibrium thermodynamics provides a coherent and self-consistent description of mitochondrial oxidative phosphorylation [see ref. 3].

An ATP/$2e^-$ ratio of 1½ does not imply that the reactions across Site 3 come to equilibrium. Cytochrome c oxidase catalyzes a concerted $4e^-$ transfer. Assuming an ATP/$2e^-$ of 1½, ΔG for the overall reaction (ATP formation and O_2 reduction) was -8 kJ/mol in the closest approach to equilibrium (static head). This is well outside the range of "near-equilibrium" (ΔG < 1.5 kJ/mol) where Onsager conditions of microscopic reversibility are valid [14].

In recent experiments, Murphy and coworkers [15] measured ΔE and Δp, the protonmotive force, for rat liver mitochondria oxidizing succinate or ascorbic acid at static head. Because $\Delta E/\Delta p$ was about 2.8 which is less than the minimum ratio of 3 predicted by an H^+/O stoichiometry of 4 (one-half a charge/O stoichiometry of 6), it was concluded that such a stoichiometry was thermodynamically impossible. This conclusion, however, may be premature in light of quantitative uncertainties concerning Δp and ΔE. Inference of Δp from equilibrium distribution of permeant probe ions requires large corrections for probe binding. Murphy and coworkers, for example, used $TPMP^+$ as a probe of $\Delta\Psi$ assuming that 67% was bound. If binding were 15% greater (i.e. 82%), Δp would be smaller and $\Delta E/\Delta p$ would equal 3 rather than being smaller. In the same work, [^{3}H]acetate was employed as a probe of ΔpH without correction for binding. If acetate binding were of the same magnitude as $TPMP^+$ binding, once again Δp would be smaller and $\Delta E/\Delta p$ would actually exceed 3. With regard to ΔE, the reduced to oxidized ratio of endogenous rather than exogenous cytochrome c is pertinent to the calculation of ΔE (and ΔG_R), since exogenous cytochrome c does not readily interact with the electron transfer sequence of intact mitochondria [16]. Similarly, an Em appropriate for bound mitochondrial cytochrome c must be employed since Em for cytochrome c in

aqueous solution is greater than that for bound mitochondrial cytochrome c [10]. With respect to the report by Murphy et al., exogenous cytochrome c was used in some experiments. In addition, the Em employed was not appropriate since it was established with reference to a mixture of both endogenous and exogenous cytochrome [17]. Thus, ΔE may have been underestimated. These experimental considerations together with speculation that localized rather than bulk phase Δp may be the driving force in chemiosmotic coupling [reviewed in ref. 18] mean that an H^+/O stoichiometry of 4 for cytochrome oxidase is not precluded on thermodynamic grounds. This conclusion is also consistent with recent measurements using very rapidly responding instrumentation showing that $H^+/2e^-$ exceeds 2 and approaches 4 in rat liver mitochondrial membranes oxidizing cytochrome c at level flow [2,19].

In the present work, reactants were measured in dilute aqueous solution in the extramitochondrial phase or from a spectral signal from endogenous cytochrome c which has been directly calibrated with reference to the standard hydrogen electrode [10]. Thus, issues of binding, equilibration and so forth do not apply for our calculations of ΔG_P, ΔE and ΔG_R. Furthermore, because the standard free energy changes of ATP formation and electron transfer across Site 3 are so great, the quantitative accuracy of measurements of ΔG_P, ΔE and ΔG_R is relatively insensitive to errors in the experimental determinations. $\Delta G_R/\Delta G_P$ across Site 3 determined here should be accurate to within a few percent. For example, a 5% error in $\Delta G_R/\Delta G_P$ would arise if one of the metabolites, say ADP, were incorrectly assayed by 250%. A comparable error would be made if the standard free energy change of ATP synthesis ($\Delta G_P'^\circ$) were in error by 3 kJ/mol or an Em in error by 25 mV. $\Delta G_P'^\circ$ and Em for oxygen and cytochrome c have been carefully evaluated, and errors of this magnitude seem unlikely.

CONCLUSION

Available evidence is insufficient to reject on equilibrium thermodynamic grounds an $ATP/2e^-$ stoichiometry of 1½ or an H^+/O stoichiometry of 4 for Site 3 of mitochondrial oxidative phosphorylation. Rather, analysis by nonequilibrium thermodynamics supports these stoichiometries.

ACKNOWLEDGMENTS

This work was supported in part by Grants HL35490 and AM37034 from the National Institutes of Health. J.J.L. is an Established Investigator of the American Heart Association.

REFERENCES

[1] Krab, K., Soos, J. and Wikstrom, M. (1984) FEBS Lett. **178**, 187-192.
[2] Lehninger, A.L., Reynafarje, B., Hendler, R.W. and Shrager, R.I. (1985) FEBS Lett. **192**, 173-178.
[3] Lemasters, J.J. (1985) Comments Mol. Cell. Biophys. **3**, 99-122.
[4] Lemasters, J.J., Grunwald, R. and Emaus, R.K. (1984) J. Biol. Chem. **259**, 3058-3063.
[5] Wanders, R.J.A., Van Den Berg, G.B. and Tager, J.M. (1984) Biochim. Biophys. Acta **767**, 113-119.
[6] Hoffman, N.E. and Liao, J.C. (1977) Anal. Chem. **49**, 2231-2234.
[7] McKee, S.A. and Fyfe, J.A. (1985) Anal. Biochem. **144**, 429-431.
[8] Lemasters, J.J. (1984) J. Biol. Chem. **259**, 13123-13130.
[9] Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta **267**, 275-299.
[10] Dutton, P.L., Wilson, D.F. and Lee, C.-P. (1970) Biochemistry **9**, 5007-5082.
[11] Clark, W.M. (1960) Oxidation-reduction Potentials of Organic Systems, Williams and Wilkins Co., Baltimore.
[12] Lemasters, J.J. and Billica, W.H. (1981) J. Biol. Chem. **256**, 12949-12957.
[13] Stucki, J.W. (1980) Eur. J. Biochem. **109**, 269-283.
[14] Van Dam, K. and Westerhoff, H.V. (1984) in Bioenergetics (Ernster, L., ed.) pp. 1-27, Elsevier, Amsterdam.
[15] Murphy, M.P., Brown, G.C. and Brand, M.D. (1985) FEBS Lett. **187**, 16-20.
[16] Wikstrom, M. and Casey, R. (1985) FEBS Lett. **183**, 293-298.
[17] Brown, G.C. and Brand, M.D. (1985) Biochem. J. **225**, 399-405.
[18] Ferguson, S.J. (1985) Biochim. Biophys. Acta **811**, 47-95.
[19] Reynafarje, B., Costa, L.E. and Lehninger, A.L. (1986) J. Biol. Chem. **261**, 8254-8262.