Effect of Pyridine Homologues on Respiratory Control and H+/O Ratio in Mitochondria*

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Yee-kin Ho and Jui H. Wang

From the Bioenergetics Laboratory, Acheson Hall, State University of New York, Buffalo, New York 14214

The effect of pyridine homologues on proton leakage, respiratory control, oxidative phosphorylation, and H+/O ratio in mitochondria have been examined. Up to a concentration of 1 mM, hydrophobic pyridine homologues diminish respiratory control in bovine heart mitochondria by increasing the State 4 respiration rate but have relatively minor effects on the State 3 and the 2,4-dinitrophenol-uncoupled respiration rates. Neither the proton gradient generated by electron transport in mitochondria in the presence of potassium ion and valinomycin, nor the rate of its anaerobic decay was affected by pyridine homologues. These observations suggest that the basal rate of electron transport is governed not directly by proton gradient, but by molecular processes in the energy-transducing membrane which can be affected by the proton gradient. By assuming that pyridine homologues are bound at low concentrations to specific functional groups in the inner membrane, the observed rates of State 4 respiration can be related quantitatively to the concentration of the organic base in solution. The observation that low concentrations of pyridine homologues decrease the H+/O ratio of mitochondria seems difficult to reconcile with the assumption that proton extrusion is driven directly by electron transport.

The molecular mechanism of mitochondrial respiratory control has been a subject of interest to many investigators ever since the discovery of the phenomenon (1). In view of the well known fact that uncoupling reagents which cause rapid leakage of protons across the mitochondrial membrane also abolish respiratory control, we should consider the possible repression of State 4 respiration by the proton gradient across the inner membrane. Previous studies of the effect of internal and external pH on the rate of light-driven electron transport in chloroplasts already suggest that the rate of basal electron transport may be controlled indirectly by the proton gradient (2, 3). In order to examine this problem further, measurements have been made in this work on the rate of proton leakage, respiratory control ratio, and P/O ratio of bovine heart mitochondria in the presence of low concentrations of pyridine or one of its more hydrophobic homologues. The experimental results also indicate that the rate of basal electron transport is not regulated directly by the proton gradient, but may be regulated by molecular processes in the inner membrane which can be affected by the pH gradient.

EXPERIMENTAL PROCEDURES

Materials—Heavy bovine heart mitochondria were prepared from fresh beef heart as described previously (4). Respiratory control ratio of the freshly prepared mitochondria was 3 to 5 with β-hydroxybutyrate as the substrate. The RCR* stayed constant for 6 h when the mitochondria were stored at 0°C. Protein concentrations were determined by the biuret method. ADP (disodium salt, grade III), ATP (disodium salt, Sigma grade), β-hydroxybutyrate, glucose, and hexokinase (from yeast, type V) were from Sigma Chemical Co. Carrier-free radioactive orthophosphate (32P) was from New England Nuclear Corp. and was found by paper chromatography to contain no detectable amount of radioactive impurity. Pyridine, 4-ethylpyridine, 4-n-butylpyridine, 4-n-butyrylpipridine, 2,6-lutidine, and pyrazine were from Aldrich Chemical Co.

Measurement of Oxygen Uptake—Oxygen uptake was measured with an oxygen electrode inserted into a thermostated and constantly stirred Gilson glass cell which was completely filled with 1.6 ml of the sample. Additions of reagents were made with Hamilton syringes through a 2-mm port in the ground glass top. The oxygen concentration was titrated with aliquots of air-saturated water.

Measurement of Respiratory Control Ratio and ADP/O Ratio—The respiratory control ratio was defined as the ratio of State 3/State 4 respiration rates and was measured as described by Estabrook (5). The ADP/O ratio, i.e. the ratio of moles of ADP phosphorylated to g-atoms of oxygen reduced, was calculated by the method of Chance and Williams (6).

Measurement of P/O Ratio—The phosphorylation reaction was carried out in the presence of glucose and hexokinase in a Gilson cell fitted with an oxygen electrode according to the procedure of Schatz and Racker (7). The reaction was initiated by the addition of ADP + 32P mixture and stopped with trichloroacetic acid (4%). The amount of radioactive glucose 6-phosphate was assayed by paper chromatography using Whatman No. 541 paper with a developing solvent of the following composition: n-butyl alcohol:n-propyl alcohol:acetone:80% (v/v) formic acid:30% (w/v) trichloroacetic acid (40:20:25:25:15) by volume plus 0.05 g of disodium salt of EDTA per 100 ml of solvent (8). The developed chromatogram was dried and cut into 1-cm strips, and the radioactivity was assayed with Aquasol in a liquid scintillation counter.

Measurement of Proton Extrusion and Anaerobic Decay of the Gradient—Proton extrusion driven by electron transport in mitochondria was monitored with a combination pH electrode (Beckman 3600) fitted to the oxygen electrode cell from the top. The outputs of the oxygen and pH electrodes were recorded by a two-channel strip-chart recorder. The reaction mixture containing 1.62 ml of reaction medium (succrose, 0.4 M; Tris-HCl, 2.5 mM, pH 7.4; KCl, 50 mM; MgCl2, 2 mM; succinate, 2 mM, β-hydroxybutyrate, 2 mM; 0.06 mg of mitochondrial protein, and valinomycin (300 µg/mg of protein) was incubated at 25°C in the closed system until anaerobiosis was reached. Then, a 100-µl aliquot of oxygen-saturated buffer was injected into the cell. The pH of the medium decreased rapidly to approach a steady state value due to the extrusion of protons by the mitochondria. After the oxygen was depleted, the steady state pH value decayed slowly back to its anaerobic level. For the conversion of observed ΔpH to nanomoles of H+ released, a calibration curve was obtained for each set of measurements by titrating the sample at the anaerobic state with 5 mM HCl. A typical experiment is shown in Fig. 1A.

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1 The abbreviation used is: RCR, respiratory control ratio.
Fig. 1 (left). Proton translocation in mitochondria. A, measurement of proton extrusion and subsequent anaerobic decay of proton gradient in bovine heart mitochondria. Experimental conditions are the same as described under "Experimental Procedures." $t_1$ indicates the time 100 µl of O$_2$-saturated buffer were added. $k_3$ represents the steady state value of proton extrusion. $t_2$ indicates the time when 2,4-dinitrophenol (30 µM) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (1 µM) was added during the anaerobic decay of the proton gradient. $k_3 k_4$ represents anaerobic decay in the presence of either uncoupler. B, kinetic analysis of the anaerobic decay of the proton gradient. The time when the system became anaerobic was taken as $t = 0$. The decay is apparently biphasic. The first order decay constants $k_1$ and $k_2$ were determined directly from the linear portions of the semilogarithmic plot. The decay constant $k_3$ was determined from the straight line obtained by subtracting the extrapolated values (- - - -) from the observed values according to the equation $\delta_t = \delta_0 \exp(-k_1 t) + \delta_1 \exp(-k_2 t)$.

FIG. 2 (right). Effect of the concentration of pyridine homologues on the rate of State 4 respiration ($R$) of bovine heart mitochondria. Each sample contained 0.56 mg of mitochondrial protein; 1.6 ml of buffer (0.4 M sucrose; 20 mM Tris-HCl, pH 7.4, 25°C; 50 mM KCl; 2 mM β-hydroxybutyrate; 2 mM MgCl$_2$ and 5 mM potassium phosphate). $O$ represents the presence of pyridine; $\bullet$, 4-picoline; $\square$, 4-ethylpyridine; $\bigcirc$, 4-t-butylpyridine; $\bigcirc$, 4-n-butylpyridine. The rate of State 4 respiration in the absence of pyridine is 45 nmol of O$_2$/mg of protein/min.

RESULTS AND DISCUSSION

The effect of hydrophobic pyridine homologues on respiratory control and oxidative phosphorylation in mitochondria is shown by the data in Table I. Pyridine homologues diminish respiratory control by increasing the rate of State 4 respiration, but have relatively only minor effects on the ADP-induced State 3 rate. That the observed increase in the basal electron transport rate is more pronounced for the more hydrophobic pyridine homologues suggests the phenomenon to be membrane-associated. On the other hand, in the absence of valinomycin, the observed ADP/O and P/O ratios remain constant. Pyridine homologues have no effect on the respiratory rate of 2,4-dinitrophenol-uncoupled mitochondria.

The data summarized in Table II show that in spite of their large effect on RCR, pyridine homologues have little effect on either the steady state value of proton extrusion driven by electron transport in the presence of potassium ion and valinomycin or the rate constants for the anaerobic decay of the proton gradient in mitochondria. These observations show that pyridine homologues do not behave as uncoupling agents which decrease RCR by dissipating the proton gradient across the inner membrane and hence suggest that the basal rate of electron transport is not regulated directly by the proton gradient, but by molecular processes in the inner membrane which can be affected either by the pH gradient or by the adsorbed pyridine homologues.

In view of these results, we may examine the effect of low concentrations of pyridine homologues on mitochondrial respiratory control more quantitatively as follows.

Let us assume that the pyridine homologue in solution is in equilibrium with that bound to specific functional groups in the inner membrane, and that micro domains of the membrane can exist in two distinct conformations with different rates $R_3$ and $R_4$ for electron transport.

Let $R = $ observed electron transport rate under State 4 conditions; $R_0 = $ observed electron transport rate in the absence of the organic base $B$; $R_1 = $ electron transport rate when these specific binding sites are completely occupied by $B$; $K$ = intrinsic equilibrium constant for the dissociation of $B$ from its specific binding sites in the membrane.

We may, as an approximation, neglect the interaction be-
Table I

Effect of pyridine homologues on respiratory control and energy coupling in heavy bovine heart mitochondria

The measurements were started by injecting mitochondria (0.66 mg of protein) into 1.6 ml of the buffered reaction medium (sucrose, 0.25 M; Tris-Cl, 20 mM, pH 7.4; 25°C; KCl, 10 mM; β-hydroxybutyrate, 2 mM; MgCl₂, 2 mM; and K₂HPO₄, 5 mM). After State 3 respiration rate was established, 300 nmol of ADP were added to obtain the State 4 respiration rate and subsequently the postphosphorylation State 4 rate. The 2,4-dinitrophenol-uncoupled respiration rate was measured by the addition of 30 μM 2,4-dinitrophenol. All respiration rates are expressed in ng-atoms of O/mg of protein/min.

| Compound and concentration (mM) | State 3 respiration rate | State 4 respiration rate | RCR | 2,4-Dinitrophenol-uncoupled respiration rate | ADP/O | P/O |
|---------------------------------|-------------------------|-------------------------|-----|---------------------------------|-------|-----|
| Control                         | 283                     | 74                      | 3.8 | 420                             | 2.84  | 3.01|
| Pyridine (pKᵢ = 5.2)b           | 1.0                     | 290                     | 83  | 3.5                             | 420   | 2.84| 2.94|
|                                | 10                      | 284                     | 142 | 2.0                             | 420   | 2.81| 3.01|
|                                | 50                      | 205                     | 205 | 2.0                             | 424   | 2.77| 2.92|
| 4-Picoline (pKᵢ = 6.1)b         | 1.0                     | 294                     | 86  | 3.4                             | 422   | 2.79| 2.95|
|                                | 10                      | 281                     | 148 | 1.9                             | 430   | 2.77| 2.87|
|                                | 25                      | 231                     | 220 | 1.1                             | 421   | 2.77| 2.92|
| 4-Ethylpyridine (pKᵢ = 6.02)b   | 1.0                     | 283                     | 98  | 2.9                             | 423   | 2.82| 3.00|
|                                | 10                      | 211                     | 163 | 1.3                             | 420   | 2.75| 2.91|
| 2,6-Lutidine                    | 5.0                     | 280                     | 152 | 1.8                             | 420   | 2.77| 2.95|
| 4-n-Butylpyridine (pKᵢ = 6.3)b  | 1.0                     | 210                     | 173 | 1.2                             | 421   | 2.77| 2.79|
| 4-n-Butylpyridine (pKᵢ = 5.99)b | 1.0                     | 260                     | 175 | 1.5                             | 428   | 2.79| 2.82|
| Pyrazine (pKᵢ = 0.65)b         | 25                      | 274                     | 74  | 3.7                             | 421   | 2.90| 2.90|
|                                | 50                      | 283                     | 79  | 3.6                             | 412   | 2.85| 2.90|

* ADP/O and P/O ratios were measured as described under “Experimental Procedures.”
* Value of pKᵢ for the conjugate acid.
* This method of measurement is inapplicable when RCR = 1.
* Not measured.

Table II

Effect of pyridine homologues on proton extrusion and the anaerobic decay of proton gradient in bovine heart mitochondria

Experimental condition and kinetic analysis are described under “Experimental Procedures.”

| Compound                        | Steady state value (θₑ) of proton extrusion | Anaerobic decay constant (s⁻¹) |
|---------------------------------|---------------------------------------------|-------------------------------|
|                                 | nmol H⁺/mg protein                          | kᵢ                           | kᵢ'                        | kᵢ''                        |
| Control                         | 165                                         | 0.057                        | 0.137                      | 0.035                        |
| Pyridine (10 mM)                | 162                                         | 0.056                        | 0.135                      | 0.034                        |
| 4-Picoline (10 mM)              | 170                                         | 0.057                        | 0.136                      | 0.036                        |
| 4-Ethylpyridine (10 mM)         | 157                                         | 0.059                        | 0.137                      | 0.036                        |
| 4-n-Butylpyridine (1 mM)        | 154                                         | 0.056                        | 0.136                      | 0.035                        |
| 4-n-Butylpyridine (1 mM)        | 153                                         | 0.058                        | 0.138                      | 0.034                        |
| Pyrazine (25 mM)                | 164                                         | 0.057                        | 0.136                      | 0.034                        |
| 2,4-Dinitrophenol (30 μM)       | 0                                           |                              |                             |                             |

Table III

Effect of pyridine homologues on the H⁺/O ratio of (K⁺ + valinomycin)-uncoupled bovine heart mitochondria

Composition of reaction medium: Tris-Cl, 1 mM, pH 7.4, 25°C; KCl, 50 mM; sucrose, 0.4 M; rotenone, 4 μM; valinomycin, 300 μg/mg of protein; mitochondria, 4 mg of protein in 1.75 ml, RCR, 3.9 with β-hydroxybutyrate. Succinate pulse, 10 μl of 0.35 M solution in the reaction medium. Electode delay: pH-electrode ≈ 0.5 s; O₂-electrode ≈ 3 s. The reduction of O₂ by succinate can be blocked completely by antimycin A both in the absence and presence of pyridine homologues.

| Proton extrusion | H⁺/O ratio | Initial rate | By analysis of first order kinetics | Steady state value | Oxygen reduction rate | By analysis of first order kinetics |
|------------------|------------|--------------|-----------------------------------|-------------------|----------------------|-----------------------------------|
|                  |            | From limiting slope | By analysis of first order kinetics | Steady state value | Oxygen reduction rate | By analysis of first order kinetics |
|                  |            | (nmol H⁺/mg protein) | (nmol H⁺/mg protein) | (ng-atoms O₂/mg protein) | (nmol H⁺/mg protein) | (ng-atoms O₂/mg protein) |
| Pyridine         |            | 10.6         | 11.2                               | 65.8              | 1.92                 | 5.5                               | 5.85                             |
|                  |            | 9.7          | 10.2                               | 75.3              | 1.83                 | 5.3                               | 5.56                             |
|                  |            | 9.9          | 10.8                               | 74.5              | 1.74                 | 5.7                               | 6.21                             |
|                  |            | 9.7          | 10.0                               | 72.0              | 1.38                 | 7.0                               | 7.27                             |
|                  |            | 9.1          | 9.2                                | 66.0              | 1.19                 | 7.6                               | 7.73                             |
| 4-Ethylpyridine  | 5.0        | 10.4         | 11.1                               | 56.3              | 1.89                 | 5.5                               | 5.85                             |
|                  | 2.5        | 9.7          | 10.0                               | 61.8              | 1.69                 | 5.7                               | 5.92                             |
|                  | 1.25       | 10.2         | 11.3                               | 70.3              | 1.55                 | 6.6                               | 7.26                             |
|                  | 0.625      | 10.5         | 11.5                               | 71.3              | 1.44                 | 7.3                               | 7.98                             |
|                  | 0          | 10.2         | 10.8                               | 72.5              | 1.32                 | 7.7                               | 8.17                             |
| 4-n-Butylpyridine| 2.5        | 9.2          | 9.4                                | 45.5              | 1.84                 | 5.0                               | 5.11                             |
|                  | 2.5        | 9.6          | 9.8                                | 71.0              | 1.53                 | 6.3                               | 6.39                             |
|                  | 0.31       | 9.8          | 10.2                               | 74.3              | 1.47                 | 6.6                               | 6.93                             |
|                  | 0          | 9.6          | 9.9                                | 67.5              | 1.23                 | 7.8                               | 7.73                             |

In order to test Equation 2, the basal respiration rates of bovine heart mitochondria were measured in the presence of a series of pyridine homologues over a range of dilute concentrations of the organic base. The results are summarized in Fig. 2.

In agreement with Equation 2, the plots of 1/(R – R∞) versus 1/[B] are within experimental uncertainties all linear with a common intercept at 1.2 × 10⁻² mg of protein-min·nmol of O₂. The dissociation equilibrium constants of pyridine homologues calculated from the corresponding slopes divided by this value are all qualitatively consistent with the hydrophobicities: 22.5 mM for pyridine, 15.5 mM for 4-picoline, 7.3 mM for 4-ethylpyridine, 3.6 mM for 4-n-butylpyridine, and 3.6 mM for 4-n-butylpyridine. These results are consistent with the assumption that bound pyridine homologues interfere with the molecular processes in the inner membrane which are responsible for respiratory control.
In order to decide whether the base form of the pyridine homologue or its conjugate acid is bound to these specific groups, similar measurements were made with mitochondrial samples containing different concentrations of 4-ethylpyridine initially at pH 6.8 and 7.8, respectively. The dissociation equilibrium constants obtained for 4-ethylpyridine are 7.6 mM and 6.8 mM at pH 6.8 and 7.8, respectively. Since these two values are almost equal within experimental uncertainties, we conclude that it is the base form of pyridine homologues (pK_a 5 to 6) which is bound to the inner membrane. Previous observation by Good (12) and Hind (13) also indicate that the free base form of amines is responsible for increasing the electron transport rate in chloroplasts. The fact that the much weaker base pyrazine (pK_a = 0.65) has no effect on respiratory control suggests that because of their higher basicity, the pyridine homologues may be hydrogen-bonded to protonated specific function groups in the inner membrane. The observed effect of pyridine homologues on the rate of State 4 respiration could not be due to their reaction as nucleophiles, because 2,6-lutidine, which is a slightly stronger base than pyridine but is a sterically hindered nucleophile, has a similar effect on the basal rate of electron transport in mitochondria as pyridine or 4-ethylpyridine.

In order to explore further the effect of pyridine homologues on the efficiency of a respiration-driven proton pump, the rates of proton extrusion and oxygen consumption by (K^+ + valinomycin)-uncoupled mitochondria were measured simultaneously in the absence and presence of various concentrations of pyridine homologues. The results are summarized in Table III, which shows clearly that the rate of oxygen reduction increases with the concentration of pyridine homologue, but that the rate of proton extrusion is essentially unchanged. Consequently, the H^+ / O ratio of (K^+ + valinomycin)-uncoupled mitochondria generally decreases as the concentration of pyridine homologues is raised. Such a conclusion seems difficult to reconcile with the assumption that proton extrusion is driven directly by electron transport (14, 15).

A question may be raised on whether the diffusion of the organic base out of respiring mitochondria could have decreased the steady state pH gradient and hence increased the respiration rate. The answer is negative, because by titrating the same mitochondria sample containing 5 mM 4-ethylpyridine during steady respiration with 10 mM NaOH and subsequently under anaerobiosis with 10 mM HCl, respectively, it was found that the buffering capacity of the medium remained the same to within 3%. This answer is expected, because the pH gradient generated by respiration is mainly due to the sharp pH rise in the small matrix volume while the pH in the large medium volume changes but slightly (see Fig. 1).

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