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Effect of Pyridine Homologues on the Basal Rate of Electron Transport and H^+/e^- Ratio in Chloroplasts[1]

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Abstract

Low concentrations of hydrophobic pyridine homologues (1 mM) were found to increase the rate of the Hill reaction in chloroplasts without significantly affecting either the steady-state proton uptake or the rate of proton leakage in the dark. By assuming that the organic base can be bound to two types of independent binding sites in the thylakoid membrane with dissociation constants K_1 and K_2 respectively, the kinetic data can be treated quantitatively. The values of K_1 and K_2 determined by the treatment are in the same relative order as the hydrophobicities of the pyridine homologues: K_1 = 1.16 mM and K_2 = 54 mM for pyridine; 0.6 and 38 mM for 4-picoline; 0.27 and 31 mM for 4-ethylpyridine, 0.10 and 4.2 mM for 4-t-butylpyridine; 0.08 and 3.2 mM for 4-*n*-butylpyridine. The rates of oxygen generation and proton uptake by illuminated chloroplasts with either ferricyanide or 1,4-benzoquinone as the electron acceptor were also measured in the presence of various pyridine homologues. Low concentration of pyridine homologues were found to decrease the H^+/e^- ratio. This last observation seems to substantiate an indirect coupling mechanism between electron transport and proton translocation.

Key Words: chloroplasts; H^+/e^- ratio; pyridine homologues; Hill reaction; coupling mechanism; electron transport; proton translocation.

Introduction

The relationship between proton gradient (ΔpH) and the rates of electron transport and photophosphorylation has been examined by different methods (Bamberger *et al.*, 1973; Portis and McCarty, 1976; Ort *et al.*, 1976). In spite of the extensive research on the control of electron transport in energy-transducing membranes, the molecular mechanism for restraining the basal

[1]Abbreviations: Chl, chlorophyll; $CF_0 \cdot CF_1$, the coupling factor complex of chloroplast; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris-(hydroxymethyl)methylglycine.

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rate of electron transport is still unresolved. Since uncouplers usually increase the basal rate of electron transport by dissipating the proton gradient, it is generally assumed that ΔpH controls the rate of electron transport (Mitchell, 1961, 1972). Permeant weak bases such as pyridine and imidazole were found to increase the rate of electron transport (Good, 1960; Hind, 1961) and steady-state proton uptake by illuminated chloroplasts (Lynn, 1968), but decrease the steady-state rate of photophosphorylation (Nelson *et al.*, 1971). The commonly accepted explanation is that permeant organic bases accumulate inside the illuminated chloroplasts and decrease the ΔpH, thereby decreasing the rate of photophosphorylation but increasing the basal rate of electron transport.

Recent studies on the respiratory control of mitochondria indicate that low concentrations of hydrophobic pyridine homologues can be bound to specific sites in the inner membrane and increase the basal rate of electron transport without affecting the ΔpH significantly (Ho and Wang, 1981a). The experimental results suggest that the basal rate of electron transport may be controlled not directly by ΔpH, but by molecular processes in the inner mitochondrial membrane which can be affected by ΔpH. In the present work, the effect of pyridine homologues on the basal rate of electron transport and the H^+/e^- ratio in illuminated broken chloroplasts has been examined.

Materials and Methods

Materials

Chloroplasts were prepared from fresh spinach leaves (Avron, 1960) and the chlorophyll content determined spectrophotometrically (Arnon, 1949). The chloroplasts were suspended in STN buffer (sucrose, 0.25 M; Tricine, 20 mM at pH 7.9; NaCl, 20 mM) at a concentration of 3 to 4 mg Chl/ml. Broken chloroplasts were obtained by 10- to 100-fold dilution of the original preparation with sucrose-free buffer at 0°C (Ho *et al.*, 1979). Pyridine homologues were found to have the same effect on fresh or frozen (stored at −70°C) broken chloroplasts.

Pyridine, 4-picoline, 4-ethylpyridine, 4-t-butylpyridine, 4-*n*-butylpyridine, and pyrazine were from Aldrich Chemical Co. Pyocyanine was from Schwarz and Mann. All other reagents were of the highest purity available.

Hill Reaction

The basal rate of electron transport in illuminated chloroplasts, from water to either ferricyanide or 1,4-benzoquinone, was monitored with a Clark type oxygen electrode as previously described (Chen and Wang, 1974). Saturating red light was provided by a 500-W projector lamp with a red filter. Prior to each measurement the reaction medium (Tricine, 20 or 2 mM at pH

7.4, 22°C; NaCl, 50 mM; sucrose, 1.2 mM; $K_3Fe(CN)_6$, 1 mM or 1,4-benzoquinone, 1 mM) was flushed with nitrogen inside a thermostated glass cell until anaerobiosis was reached. Then the chloroplast sample was added and incubated in the dark for 15 min before the actinic red light (250 J m^{-2} sec^{-1}) was turned on by means of a shutter and the rate of oxygen generation was subsequently monitored. The change in oxygen concentration in the cell was determined by titration with air-saturated water.

Proton Uptake and H^+/e^- Ratio

Proton uptake by illuminated chloroplasts driven by cyclic electron transport with pyocyanine as the mediator, and driven by noncyclic electron

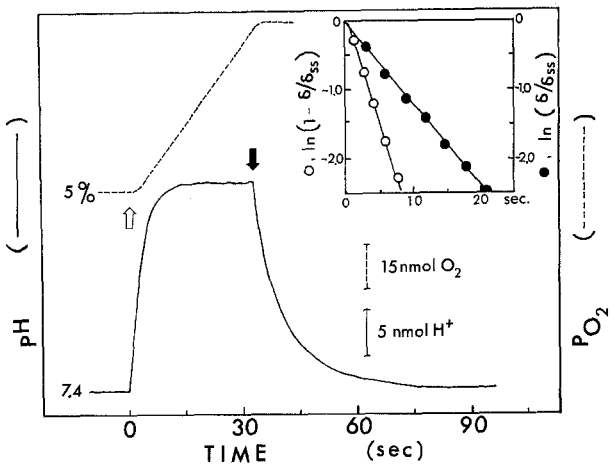

Fig. 1. Oxygen generation and proton uptake by illuminated chloroplasts with 1,4-benzoquinone as the electron acceptor. The reaction medium (Tricine 2 mM pH 7.4; NaCl 50 mM; 1,4-benzoquinone 1 mM; sucrose 1.2 mM) was flushed inside the thermostated cell with nitrogen. After anaerobiosis was reached, the thawed frozen chloroplasts were added to a given concentration of 80 μg/ml and the sample was incubated anaerobically in the dark for exactly 15 min at 25°C. Both pH and oxygen concentration had reached a constant value during the incubation. Subsequently the actinic red light (250 J m^{-2} sec^{-1}) was turned on (⇑) and the rates of oxygen generation and proton uptake were monitored simultaneously. When the medium pH reached a steady value, the light was turned off (⇓) and the medium pH was allowed to decay back to its initial value. In the insert, O represents the value ln $(1 - \delta/\delta_{ss})$ during illumination, where δ and δ_{ss} are nanomoles of proton taken up from the medium at time t and at steady state respectively; ● represents the value of ln (δ/δ_{ss}) after the light was turned off. The slopes of these linear plots give the first-order rate constant for the decay of proton gradient under illumination (k_L) and in the dark (k_D) respectively, and the initial rate (R_0) of proton uptake is equal to $k_{Lss}{}^{\delta}$, as described in Materials and Methods.

transport with 1,4-benzoquinone as the acceptor, respectively, was measured by means of a combination pH-electrode (Beckman 39030) fitted to the thermostated glass cell. Proton translocation and oxygen generation were measured and recorded simultaneously. In order to convert the observed ΔpH to nmols H^+ taken up, a calibration curve was obtained for each set of measurements by titrating the sample under illumination with 5.00 mM HCl.

The initial rate of proton uptake was determined both graphically from the limiting slope and by first-order kinetic analysis (Ho *et al.*, 1979) according to the equation $\ln (1 - \delta/\delta_{ss}) = -k_L t$, where δ and δ_{ss} denote nmols of H^+ taken up from the medium per milligram Chl at time t and at the steady state respectively, and k_L is a first-order rate constant which is independent of t and δ but varies with light intensity. Differentiation of the above equation with respect to t gives the rate equation $d\delta/dt = k_L(\delta_{ss} - \delta)$, which reduces to $R_0 = k_L\delta_{ss}$ at $t = 0$, where R_0 represents the initial rate of proton uptake when the actinic light was first turned on. Consequently, quite precise values of R_0 were obtained by dividing the slopes of the linear $\ln (1 - \delta/\delta_{ss})$ vs. t plots by the corresponding values of δ_{ss} as illustrated in Fig. 1.

Similarly, the outward leakage of the accumulated protons in the dark was found to obey the first-order decay equation $\ln (\delta/\delta_{ss}) = -k_D t$, where k_D represents the first-order leakage constant in the dark, which was also determined quite precisely from the corresponding linear plot in Fig. 1.

Results and Discussion

The observed effects of pyridine homologues on the rate of the Hill reaction with ferricyanide as the electron acceptor and the steady-state proton uptake under the conditions of cyclic electron transport by illuminated chloroplasts are summarized in Table I. The data show that pyridine homologues increase the basal rate of electron transport and steady-state proton uptake, but decrease the first-order rate constant (k_D) for the leakage of accumulated protons across the thylakoid membrane in the dark. It is interesting to note that at the same concentration, the more hydrophobic organic bases cause a greater increase in the rate of the Hill reaction. Indeed, 4-*n*-butylpyridine at 1 mM caused a larger increase than pyridine at 50 mM, even though the latter produced a much larger δ_{ss}.

Effect on Hill Reaction

In order to examine this effect more quantitatively, let us assume that pyridine homologues can be bound to two types of sites in the thylakoid

Table I. Effect of Pyridine Homologues on the Hill Reaction and Proton Uptake in Fresh Broken Chloroplasts[a]

Compound and concentration	Rate of noncyclic[b] electron transport (μmol O_2/mg Chl/hr)	Steady-state proton uptake, δ_{ss} (μmol H^+/mg Chl)[c]	k_D (sec^{-1})[d]
Control	95	1.05	0.081
Pyridine (pK_a = 5.2)			
10 mM	149	3.98	0.048
25 mM	161	4.58	0.045
50 mM	179	6.28	0.042
4-Picoline (pK_a = 6.1)			
10 mM	188	4.52	0.052
25 mM	215	4.61	0.061
4-Ethylpyridine (pK_a = 6.02)			
1 mM	130	2.79	0.079
10 mM	196	4.69	0.051
4-*t*-Butylpyridine (pK_a = 5.99)			
1 mM	211	3.20	0.082
4-*n*-Butylpyridine (pK_a = 6.0)			
1 mM	210	2.82	0.083
Pyrazine (pK_a = 0.65)			
30 mM	94	1.04	0.082

[a] Several values in this table are taken from Ho and Wang (1982). The experimental conditions of of each measurement are described in Materials and Methods.
[b] Potassium ferricyanide was used as the electron acceptor.
[c] Under cyclic electron transport conditions with pyocyanine as the electron mediator.
[d] k_D is the first-order rate constant for the decay of proton gradient in the dark.

membrane with dissociation equilibrium constants K_1 and K_2 respectively and that $K_1 \ll K_2$. Let R be the observed basal rate of electron transport in the absence of organic base B, R_1 be the rate when all of the K_1 sites but none of the K_2 sites are occupied by the organic base B, and R_2 be the rate when both the K_1 and the K_2 sites are completely occupied by B.

Let us consider the thylakoid membrane to be subdivided into a large number of microdomains. The fraction of domains with neither type of binding sites occupied by B is $K_1/(K_1 + [B] + [B]^2/K_2)$, that with both types of binding sites occupied by B is $[B]/(K_2 + [B] + K_1K_2/[B]$, and that with all of its K_1 sites occupied but none of its K_2 sites occupied is $1/(K_1/[B] + 1 + [B]/K_2)$. Therefore the experimentally observed basal rate of electron transport R' in the presence of pyridine homologue can be expressed as

$$R' = R\left(\frac{K_1}{K_1 + [B] + [B]^2/K_2}\right) + R_1\left(\frac{1}{K_1/[B] + 1 + [B]/K_2}\right) + R_2\left(\frac{[B]}{K_1K_2/[B] + K_2 + [B]}\right) \quad (1)$$

At low concentrations of B when [B] $\ll K_2$, Eq. (1) reduces to

$$\frac{1}{R' - R} = \left(\frac{1}{R_1 - R}\right)\left(1 + \frac{K_1}{[B]}\right) \tag{2}$$

At higher concentrations of B when [B] $\gg K_1$, Eq. (1) becomes

$$\left(\frac{1}{R' - R_1}\right) = \left(\frac{1}{R_2 - R_1}\right)\left(1 + \frac{K_2}{[B]}\right) \tag{3}$$

The basal rate of electron transport was measured over a wide range of concentrations of different pyridine homologues, and the experimental values of $1/(R' - R)$ are plotted against the reciprocals of concentration of the respective pyridine homologues in Fig. 2. The linear plots in Fig. 2 seem biphasic in nature. Extrapolation of the straight lines in the low concentration range to infinite concentration gave a common intercept $I = 0.0168 \pm 0.003$ mg Chl/hr/μmol O_2 as predicted by Eq. (2). Since $R = 84$ μmol O_2/mg Chl/hr, we obtain, from $I = 1/(R_1 - R)$, $R_1 = 144$ μmol O_2/mg Chl/hr. The values of the dissociation equilibrium constant K_1, computed by dividing the respective slopes of the straight lines in Fig. 2 by the above value of their common intercept I, were found to be in the same relative order as the approximate hydrophobicities of the pyridine homologues: $K_1 = 1.16$ mM for

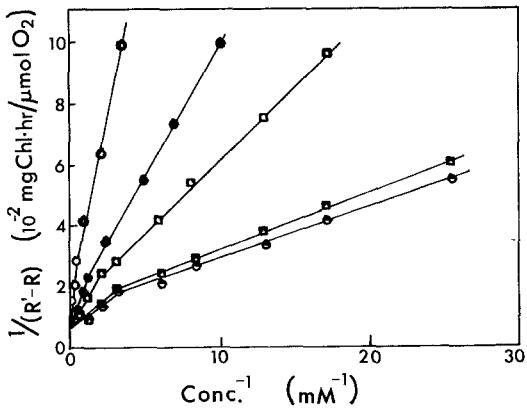

Fig. 2. Dependence of the increase in the Hill reaction rate on the concentration of pyridine homologues. ○ represent value in the presence of pyridine: ●, 4-picoline; □, 4-ethylpyridine; ◪, 4-*t*-butylpyridine, and ◓, 4-*n*-butylpyridine. Chlorophyll concentration, 75 μg/ml; Tricine, 50 mM, pH 7.4, at 25°C; NaCl, 50 mM; sucrose, 0.25 M, potassium ferricyanide, 2 mM. The intensity of the actinic red light was 250 J m^{-2} sec^{-1}. Rate of oxygen evolution in the absence of pyridine homologue = 84 μmol O_2/mg chlorophyll/hr.

pyridine, 0.6 mM for 4-picoline, 0.27 mM for 4-ethylpyridine, 0.1 mM for 4-*t*-butylpyridine, and 0.08 mM for 4-*n*-butylpyridine.

The experimental value of $1/(R' - R - 1/I) = 1/(R' - R_1)$ for the higher concentrations of pyridine homologues are plotted against 1/[B] in Fig. 3. As predicted by Eq. (3), all straight lines extrapolate to a common intercept which gives the value $R_2 = 234$ μmol O_2/mg Chl/hr. The values of the dissociation equilibrium constant K_2 computed from the respective slopes and the common intercept are larger than the corresponding values of K_1 but still consistent with the approximate hydrophobicities: 54 mM for pyridine, 38 mM for 4-picoline, 31 mM for 4-ethylpyridine, 4.1 mM for 4-*t*-butylpyridine, and 3.2 mM for 4-*n*-butylpyridine.

It is of interest to note that the value of R_2 which represents the maximum rate of basal electron transport in the presence of pyridine homologues is approximately equal to only 1/2 of the uncoupled electron transport rate (440 μmol O_2/mg Chl/hr) obtained in the presence of 5 μM FCCP or 2 mM ammonia. This observation suggests that the mechanism for increasing the basal rate of electron transport by pyridine homologues is different from that by uncouplers which reduce ΔpH by accelerating the leakage of accumulated protons.

Recent studies in this laboratory showed that pyridine homologues can also decrease the steady-state rate of photophosphorylation and light-stimulated ATPase activity. When these latter effects were treated quantitatively by the same method as described above, the dissociation equilibrium constants obtained in the higher concentration range were found to be of similar magnitude as the corresponding K_2 values determined in the present work (Ho and Wang 1982). These results suggest that the basal rate of

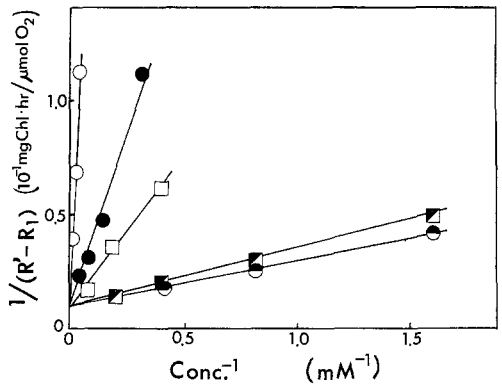

Fig. 3. Analysis of the increase in the Hill reaction rate caused by higher concentrations of pyridine homologues according to Eq. (3). The experimental conditions are the same as in Fig. 2.

Table II. Effect of Pyridine Homologues on the Observed H^+/e^- Ratio in Broken Chloroplasts[a]

Concentration (mM)	Rate of O_2 generation (ng-atom/mg Chl/sec)[e]	Steady-state proton uptake δ_{ss} (nmol H^+/mg Chl)[f]	Initial rate of H^+ uptake, R_0 (nmol H^+/mg Chl/sec)		k_D (sec^{-1})[d]	H^+/e^- ratio
			From the limiting slope[b]	By first-order kinetic analysis[c]		
			Pyridine			
0	27.5	190	65.3	66.2	0.110	1.21
1.25	29.2	273	56.2	57.2	0.102	0.98
2.5	31.2	370	53.1	54.9	0.094	0.87
5.0	35.1	470	53.1	54.2	0.081	0.77
10.0	44.2	540	51.2	52.0	0.072	0.59
			4-Picoline			
0	27.1	182	64.0	64.0	0.113	1.18
0.625	29.6	220	55.2	56.0	0.112	0.95
1.25	34.2	278	53.1	54.1	0.110	0.79
2.5	37.6	326	51.5	52.1	0.098	0.69
5.0	40.2	475	50.1	50.1	0.086	0.62
			4-Ethylpyridine			
0	27.5	185	62.6	63.8	0.116	1.16
0.313	29.6	192	59.8	60.1	0.116	1.02

0.625	33.5	235	59.2	59.5	0.110	0.89
1.25	38.2	270	56.8	57.2	0.097	0.75
2.5	41.5	331	56.1	56.5	0.093	0.68
			4-*t*-Butylpyridine			
0	28.0	187	65.2	65.5	0.114	1.17
0.156	35.2	199	63.8	64.6	0.115	0.92
0.313	39.0	217	62.2	63.1	0.115	0.81
0.625	43.2	235	61.0	61.8	0.116	0.72
1.25	49.4	275	60.0	60.2	0.116	0.61
			4-*n*-Butylpyridine			
0	27.2	191	65.9	66.2	0.118	1.20
0.156	37.1	199	64.0	64.8	0.118	0.87
0.313	40.1	210	63.1	63.9	0.119	0.79
0.625	44.1	233	63.1	63.1	0.118	0.72
1.25	51.0	276	62.0	62.1	0.119	0.61

[a] Composition of the reaction medium: 20 mM Tricine, pH 7.4, 22°C; 50 mM NaCl; 1.2 mM sucrose; 1 mM 1,4-benzoquinone. Light intensity: 250 J m^{-1} sec^{-1}. The experimental procedure is summarized in the caption of Fig. 1. Frozen chloroplasts from the same batch were used for the study of each pyridine homologue.

[b] Determined graphically from the limiting slope of the experimental trace.

[c] Calculated from the linear plot of ln $(1 - \delta/\delta_{ss})$ versus t after the light was turned on, as illustrated in Fig. 1.

[d] Calculated from the linear plot of ln (δ/δ_{ss}) versus t after the light was turned off, as illustrated in Fig. 1.

[e] For comparison with values in Table I, the oxygen generation rate in the absence of pyridine homologues is 50 μmol O_2/mg Chl/hr.

[f] The steady-state proton uptake under conditions of noncyclic electron transport as described in the caption of Fig. 1.

electron transport and functions of the $CF_0 \cdot CF_1$ complex may be regulated by the same molecular process in the thylakoid membrane.

Effect on H^+/e^- Ratio

According to the chemiosmotic hypothesis (Mitchell, 1966, 1976), proton pumping is driven directly by proton loops of the electron transport chain with a fixed H^+/e^- ratio. On the other hand, the indirect mechanism assumes that proton pumping is driven by electron transport through redox-dependent conformation changes in the energy-transducing membrane (Boyer, 1975; Papa, 1976; Chance, 1977), and hence may allow a decrease in the H^+/e^- ratio if these conformation changes are hindered by the binding of pyridine homologues.

The effect of pyridine homologues on the observed H^+/e^- ratio in illuminated chloroplasts was examined by using 1,4-benzoquinone as the electron acceptor and water as the electron donor so that the protons generated by water oxidation will be taken up by benzoquinone reduction, and hence the net proton translocation due to noncyclic electron transport can be evaluated accurately. Figure 1 illustrates a typical experiment in which proton translocation and electron transport were measured simultaneously. The experimental trace shows a reversible proton uptake with no net proton uptake or release in the overall chemical reaction. Moreover, it was found that under the experimental conditions both electron transport and proton uptake were inhibited by DCMU (5 μM). Consequently we may conclude that the observed proton uptake was due entirely to noncyclic electron transport. The measured H^+/e^- ratios in the presence of various concentrations of pyridine homologues are summarized in Table II which shows that even low concentrations of hydrophobic pyridine homologues increase the basal rate of electron flow without a proportionate increase in the rate of proton uptake. Consequently the observed H^+/e^- ratio decreases as the concentrations of pyridine homologues are increased.

The electrons which reduce benzoquinone may come directly from PSII passing through one proton pumping site, or from PSII via PSI passing through two proton pumping sites. The observed H^+/e^- ratio in the absence of pyridine homologues suggests that under these experimental conditions benzoquinone functions mainly as a direct electron acceptor from PSII. The observed substantial decrease in the H^+/e^- ratio by millimolar concentrations of hydrophobic pyridine homologues is probably due to their interference with the proton-pumping mechanism, not due to the switching of electron-accepting sites for benzoquinone which would only increase the H^+/e^- ratio. This interpretation is also consistent with the observation that low concentrations of hydrophobic pyridine homologues do not decrease the

efficiency of two-stage photophosphorylation (Ho and Wang, 1981b), i.e., these organic bases do not act as uncouplers. These experimental results seem to substantiate an indirect mechanism for proton pumping.

Acknowledgments

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