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EPR STUDIES OF HIGHER PLANT MITOCHONDRIA

II. CENTER S-3 OF SUCCINATE DEHYDROGENASE AND ITS RELATION TO ALTERNATIVE RESPIRATORY OXIDATIONS

PETER R. RICH and WALTER D. BONNER Jr.

Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Penn. 19174 (U.S.A.)

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Summary

1. An electron paramagnetic resonance study of the high potential iron sulfur (HiPIP-type) Center S-3 of higher plant mitochondria is described. This center is the major HiPIP-type center associated with plant mitochondria and it displays physical properties which are similar to its mammalian counterpart. It has a pH-independent midpoint potential of +65 ± 10 mV between pH 6.0 and 8.5.

2. The behavior of Center S-3 in a variety of steady-state conditions suggests that it is of physiological significance in electron transport. Furthermore, it can be shown that the alternative oxidase, which is present in many higher plant mitochondria, tends to keep this center oxidized in the presence of succinate and cyanide. This indicates that the alternative oxidation site is on the electron-donating side of the Center S-3.

3. Salicylhydroxamic acid, an inhibitor of the alternative pathway, does not affect the midpoint potential, signal size or shape, or temperature and power saturation profiles of Center S-3, suggesting that direct autoxidation of this center cannot account for alternative oxidase activity. This is further confirmed by the finding that the presence of succinate dehydrogenase is not necessary for alternative oxidase activity with NADH as respiratory substrate in submitochondrial particles.

Abbreviation: HiPIP is an abbreviation for a class of iron sulfur proteins which are paramagnetic in their oxidized form. The abbreviation is derived from the first of this type to be detected, which was termed a high potential iron sulfur protein [1]. The use of the nomenclature in this manuscript does not imply any redox information.

Introduction

A group of iron sulfur centers exist which are paramagnetic in their oxidized forms, in contrast to the ferredoxin-type centers. They are analogous to the bacterial high potential iron sulfur proteins [1] and are called HiPIP-type centers. Two such proteins have been reported to occur in mammalian mitochondria. The first of these is Center S-3 of succinate dehydrogenase, which is readily detected in aerobic mitochondria or in reconstitutively active succinate dehydrogenase preparations [2—4]. A second HiPIP-type center, termed "Ruzicka's center" or "Center bc-3" [5,6], has also been found in mammalian mitochondria, although it was not present in submitochondrial particle preparations. These centers have been well characterized by electron paramagnetic resonance [2—6].

We have already described the presence of HiPIP-type Center S-3 in a variety of higher plant mitochondria [7,8]. The present report extends these observations. Center S-3 had a midpoint potential of +65 ± 10 mV and this was pH independent between pH 6.0 and 8.5. Studies of temperature and power saturation parameters and potential measurements indicated that the second HiPIP-type center [5] was not present or in low amounts. Quantitation of the Center S-3 revealed that it was present in amounts close to the amount of cytochrome aa_3.

The alternative oxidation pathway, which is present in many higher plant mitochondria [9], is located on the substrate side of the classical coupling site II of the respiratory chain. Since Center S-3 donates electrons to this region of the chain, it was of interest to study its relation to the alternative oxidation step. Evidence from steady-state experiments is presented to show that the alternative oxygen consumption step is past the electron-donating side of Center S-3. Furthermore, salicylhydroxamic acid, an inhibitor of the alternative oxidase, did not affect the midpoint potential, temperature or power saturation profile, or signal size or shape of Center S-3, suggesting that direct autoxidation of this component cannot account for alternative oxidase activity. This deduction is further confirmed by that finding that alkaline-washed submitochondrial particles of *Sauromatum guttatum* spadix retained cyanide-insensitive oxygen consumption with NADH as respiratory substrate, even though they had lost most of their succinate oxidase activity and EPR-detectable Center S-3.

The relevance of these studies to notions of the location and nature of the alternative pathway is discussed.

Materials and Methods

Plant materials. Etiolated mung bean hypocotyls (*Phaseolus aureus*) were grown for 5 days in a dark room maintained at 28°C and 60% relative humidity. *Symplocarpus foetidus* (Eastern skunk cabbage) inflorescences were collected from swamp areas in Pennsylvania in February and March and *Sauromatum guttatum* inflorescences were grown indoors from dry corns. Tulip bulbs (*Tulipa gesnerana* var. Darwin) and potato tubers (*Solanum tuberosum*) were purchased locally.

Preparation of mitochondria and submitochondrial particles. Mitochondria were prepared as described by Bonner [10]. In the case of *Sa. guttatum* and *Sy. foetidus* spadices, EDTA concentration was doubled to 2 mM in the homogenisation medium and bovine serum albumin was increased to 0.5% (w/v) in both homogenisation and resuspension media.

Previously described methods of preparation of higher plant submitochondrial particles [11—13] were found to be inadequate in terms of both yield of protein and activity of electron transport. Instead, a new method was developed which produced superior yield and activity of material. Washed mitochondria (generally 50—100 mg protein/ml) were diluted to ten times their original volume slowly with ice-cold distilled water to which had been added 0.3 mM ATP and 1 mg/ml bovine serum albumin. This produced a final osmolarity of the suspension of 35 mosM, excluding any contribution from the mitochondrial protein or from the bovine serum albumin. The suspension was then French-pressed at 3000 lb/inch^2. An initial centrifugation at 7000 $\times$ g_{av} for 10 min removed unbroken or coagulated particles. The supernatant was then centrifuged at 150 000 $\times$ g_{av} for 30 min to precipitate the submitochondrial particles. These formed a translucent tight reddish pellet which was resuspended in a small volume of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate at pH 7.2, to a final protein concentration of generally between 15 and 50 mg/ml. A more detailed account of the properties of submitochondrial particles prepared by this method will be presented elsewhere.

EPR measurements. Samples for EPR measurements of mitochondria in various metabolic states were frozen rapidly in an isopentane/methylcyclohexane (5 : 1, v/v) freezing mixture (81°K), and stored in liquid nitrogen until assayed.

All spectra were obtained with a Varian E-4 or E-109 EPR spectrometer (Varian Associates). The temperature of the samples for EPR measurements was controlled with a variable temperature cryostat (Air Products Model LTD-3-110). Temperature was monitored with a carbon resistor placed in the helium flow directly below the sample and g values were corrected by reference to a weak pitch standard [14]. Quartz glass EPR sample tubes were calibrated with a standard copper sulphate/EDTA solution and values for signal heights were corrected correspondingly.

Relative quantitation was performed by comparison of the double integrals of the appropriate signals which had normally been signal averaged. Signal averaging and double integrations were carried out with a Nicolet signal averager (NIC-1074). Absolute quantitation was carried out in the same manner by comparison of the double integral of the signal to the double integral of a 25 mM copper sulphate/EDTA standard under identical conditions. In all cases, signals were checked to ensure that they were not saturating at the powers and temperatures used.

Spectrophotometric and cytochrome measurements. Difference spectra were produced with a Johnson Research Foundation split beam spectrophotometer. The cytochrome content was estimated from a succinate reduced (anaerobic) minus aerobic (no substrate) difference spectrum. The extinction coefficients used were those of Chance and Williams [15] although the wavelengths of measurements were adjusted so that they were optimal for the plant system.

Redox potentiometry. Redox titrations were performed with an anaerobic vessel under a constant flow of argon as described by Dutton [16]. Potentials were measured with a platinum electrode against a calomel standard. Redox mediators are given in the text. Plant mitochondria generally possess endogenous reductant which is difficult to remove and which causes a constant drift to lower potentials. To minimise this problem, the mitochondria were diluted to approx. 10 mg/ml in 0.3 M mannitol, 5 mM $MgCl_2$ and 10 mM KCl and stirred while pure oxygen was blown across the surface for 1—2 h before the redox titration was performed. The appropriate buffer was added before the titration. This problem did not exist with submitochondrial particles. Aliquots of sodium ascorbate, $Na_2S_2O_4$ or $K_3Fe(CN)_6$ were used to lower or raise the potential of the system when necessary.

Oxygen consumption. Oxygen consumption was measured with mitochondria, or submitochondrial particles resuspended to an appropriate protein concentration in a medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate at pH 7.2. A Clark-type oxygen electrode, manufactured by Hansatech Ltd. was used for the measurements.

For inhibitor studies, the mitochondria were always preincubated with inhibitor before substrate was added so that maximum inhibition was obtained.

Steady-state experiments. Steady-state experiments were carried out with oxygen-saturated medium A (0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate at pH 7.2) as resuspension medium. The concentrations of reagents added were succinate, 10 mM; NADH, 1 mM; malate, 20 mM; ATP, 0.3 mM; ADP, 0.3 mM; KCN, 1 mM; antimycin A, 1 μg/ml; salicylhydroxamic acid, 1 mM; dinitrophenol, 20 μM. Enough time was allowed (generally 10—15 s for 3 mg/ml protein) so that equilibrium had been reached but anaerobiosis had not begun. Oxygen was blown over the surface of the liquid during the incubation, after which the sample was transferred to a quartz EPR tube and frozen rapidly in an isopentane/methylcyclohexane (5 : 1, v/v) freezing mixture at 81°K.

The salicylhydroxamic acid stock solution was 5 mM in medium A, dinitrophenol was 2 mM in medium A and both were in the absence of organic solvents.

Others. Standard chemicals used were of the highest grade available commercially. Protein was measured by the method of Lowry et al. [17] with crystalline bovine serum albumin as a standard.

Results

The detection and quantitation of Center S-3 in higher plant mitochondria

At low temperature (8°K) and moderate microwave power (5 mW) a first derivative EPR signal characteristic of high potential iron sulfur Center S-3 (HiPIP-type Center S-3) was detected in all mitochondria examined. The signal was also found in submitochondrial particles. In many instances, overlapping signals caused by an interacting pair of ubisemiquinone species [8] tended to obscure the signal, although these overlapping signals were very labile [8] and were completely absent from submitochondrial particles. A further contribution to the total spectrum was a small free radical signal centered at $g = 2.00$,

which was partially saturated at these temperatures and powers. By raising the temperature and lowering the power, this signal alone (although still partially saturated) could be observed. This is illustrated in Fig. 1 for mung bean submitochondrial particles in the aerobic (no substrate) state. It can be seen that as the temperature was raised above 10°K, the g = 2.018 peak (Center S-3) diminished rapidly, causing the feature centered at g = 2.004 to become much more prominent. A detailed characterization of this unsplit g = 2.00 component, which is attributed to a semiquinone species, will be presented elsewhere. The ratio of Center S-3 to the unsplit g = 2.00 component was very variable between mitochondria from different plant sources yet was fairly consistent within different preparations from the same tissue.

The first derivative signal attributed to Center S-3 was centered at g = 2.01, peaked in all mitochondria examined at g = 2.018 ± 0.001, and had a peak to peak width of approx. 25 G. These properties are rather similar to the analogous mammalian mitochondrial component [2–6].

Quantitation of the Center S-3 signal was attempted by double integration and by comparison to the double integral of a copper sulphate/EDTA standard of known concentration under the same (non-saturating) conditions. The signal caused mostly by Center S-3 (together with some contribution from the g = 2.00 free radical signal) was obtained by addition of 0.1% (v/v) ethanol to those mitochondria in which the spin-spin interaction of the semiquinone species was also normally present (see ref. 8). The results of such quantitation are presented in Table I and indicate that, in the case of mung bean mitochondria,

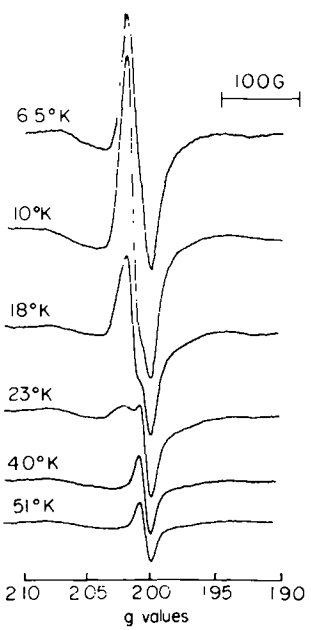

Fig. 1. First derivative EPR spectra of oxidized mung bean submitochondrial particles. Submitochondrial particles of mung beans were resuspended in medium A [10] to a final protein concentration of 26 mg/ml and 500 μM $K_3Fe(CN)_6$ was added. The sample was then frozen rapidly in liquid nitrogen. Conditions of measurement were: modulation amplitude, 12.5 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.102 GHz; gain, 10^3.

TABLE I

THE STOICHIOMETRIC RELATION BETWEEN CENTER S-3 AND THE CYTOCHROME COMPONENTS IN HIGHER PLANT MITOCHONDRIA

Center S-3 was assayed by double integration of the first derivative EPR signal and comparison to a copper/EDTA standard. Cytochromes were assayed from a succinate (anaerobic) versus no substrate (aerobic) difference spectrum. Details of these methods are presented in the text.

Tissue source of mitochondria	Center S-3	Cytochromes		
		b	$c_1 + c$	$a + a_3$
Sa. guttatum spadix	0.074	0.08	0.12	0.038
Mung bean (*Phaseolus aureus*) hypocotyl	0.072	0.11	0.22	0.14

there is approximately one Center S-3 per two respiratory chains (measured from a room temperature succinate anaerobic minus aerobic (no substrate) difference spectrum), assuming that there is one cytochrome oxidase per respiratory chain. It should be noted that the Center S-3 measurements are a slight overestimation since some extra component (either as a $g = 2.00$ component or some residual split $g = 2.00$ signal [8]) is always present. Quantitation at an optimal temperature and power for Center S-3 (8—10°K and 2 mW power) minimizes this overestimation.

In the case of *Sa. guttatum* mitochondria, which are highly cyanide insensitive and which have a lower level of cytochrome oxidase than normal mitochondria, the Center S-3 is actually in excess of cytochrome oxidase (Table I).

Temperature dependencies

The temperature dependency of the HiPIP-type Center S-3 signal in mung bean mitochondria was examined in the presence of 0.1% (v/v) ethanol (i.e. in the absence of the split semiquinone signals, see ref. 8) and in the presence of 2 mM salicylhydroxamic acid plus 0.1% (v/v) ethanol (an inhibitor of the alternative respiratory pathway [18]). No significant differences in the temperature profiles of the two samples could be observed, indicating that salicylhydroxamic acid did not significantly alter the relaxation properties of Center S-3.

As the temperature was raised above 10°K, the $g = 2.018$ peak height dropped off sharply and the profile of this was rather similar in both mitochondria and submitochondrial particles of mung bean hypocotyls. Also of significance in this respect is the fact that the peak position at $g = 2.018$ did not move to higher g values (lower field positions) as the temperature was raised. These results are taken as evidence that the Ruzicka center [5] is in very low levels or is absent from these mitochondria. This is not wholly surprising since the center is probably not of physiological significance in the mammalian mitochondrial respiratory chain [6].

In a similar experiment performed with *Sa. guttatum* spadix mitochondria, it was found that as the temperature of an aerobic sample of mitochondria was raised, a second peak at a g value of approx. 2.03 did appear, first causing an apparent broadening of the $g = 2.018$ (Center S-3) peak, and then producing a distinct peak alongside the Center S-3 peak (see Fig. 2). The $g = 1.985$ trough

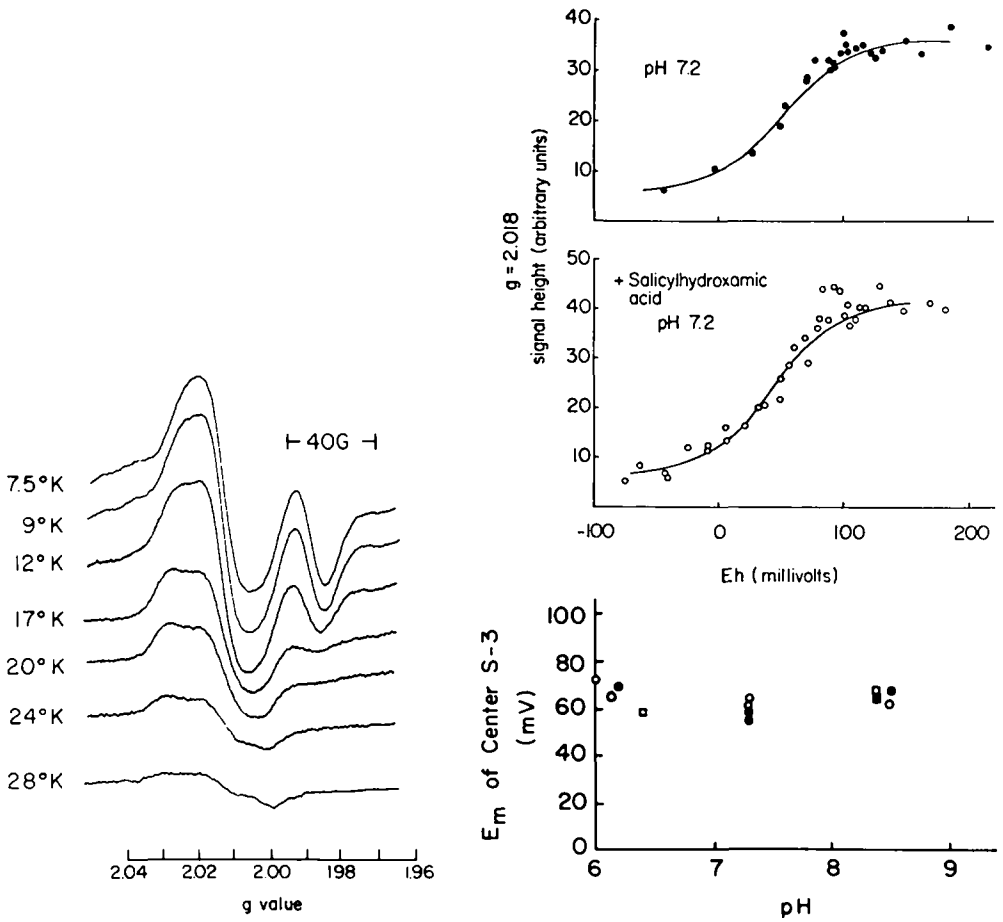

Fig. 2. Temperature dependency of oxidized *Sa. guttatum* spadix mitochondrial EPR spectrum. A sample of oxygenated *Sa. guttatum* mitochondria (approx. 20 mg protein/ml) was frozen rapidly in a quartz EPR tube. First derivative EPR spectra were then recorded at various temperatures under the following conditions: modulation amplitude, 12.5 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.18 GHz; scan range, 400 G.

Fig. 3. Potentiometric titration of mung bean Center S-3. Mitochondria or submitochondrial particles were resuspended in a medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and either 50 mM 2-(*N*-morpholino)-ethane sulfonic acid (for pH range 6.0—6.5), 10 mM potassium phosphate (for pH range 7.0—7.2) or 50 mM Tris · HCl (for pH range 8.0—8.5). The whole was made anaerobic with argon and an anaerobic redox titration was performed as described by Dutton [16] in the presence of 25—50 μM each of the following mediators (E_m at pH 7.0 given in parentheses): *N*-ethyl phenazonium ethosulphate (+55 mV), *N*-methyl phenazonium methosulphate (+8 mV), diaminodurol (+250 mV), pyocyanine (—40 mV), duroquinone (+10 mV), and 2-hydroxy-1,4-naphthoquinone (—125 mV). Samples were injected anaerobically into quartz EPR tubes, frozen rapidly in a methylcyclohexane/isopentane (81°K) freezing mixture, and stored at 77°K until assayed. Conditions of EPR measurement were: modulation amplitude, 12.5 G; modulation frequency, 100 kHz; temperature 8°K. The lines are theoretically calculated Nernst curves for an $n = 1$ redox reaction. In the figure of pH dependency, the symbols are: ○, mitochondria; ●, mitochondria plus 1 mM salicylhydroxamic acid; □, submitochondrial particles; ■, submitochondrial particles plus 1 mM salicylhydroxamic acid.

that can be seen in Fig. 2, together with its associated features, has the same temperature profile as Center S-3 and has been discussed in detail in ref. 8. The $g = 2.03$ feature did not appear in submitochondrial particles prepared from the

same tissue and was attributed to the Ruzicka (bc-3) center which is present only in small amounts (no more than 25% of the amount of Center S-3) in these mitochondria and which is in even lower amounts or is absent from intact mitochondria prepared from mung beans. It was found that 1 mM salicylhydroxamic acid did not affect the signal size, shape, or temperature dependency of the *Sauromatum* Ruzika-type center. In neither mung bean nor *Sa. guttatum* mitochondria did 1 mM salicylhydroxamic acid affect the power saturation profile of the HiPIP signal at 8 or 15°K.

The midpoint potential and pH dependency of Center S-3

An anaerobic redox titration of mitochondria in the presence of a range of mediators was carried out [16] to determine the midpoint potential of Center S-3. The results of some typical titrations of mung bean mitochondria at pH 7.2 in the presence and absence of salicylhydroxamic acid are shown in Fig. 3 and from these experiments the midpoint potential of Center S-3 was +65 ± 10 mV. The curves drawn through the points are theoretical curves for $n = 1$ redox changes. Further titrations were performed at pH values between 6 and 8.5 and no significant deviation from this midpoint value was obtained. The midpoint potential of Center S-3 was also determined in the presence of salicylhydroxamic acid, and this compound did not alter the value of the midpoint of the titration between pH 6.2 and 8.5. The midpoint potential of the center in submitochondrial particles of mung beans was in a similar range to that obtained with whole mitochondria, demonstrating that the endogenous reductants of the mitochondria were not interfering with the titration. The accumulated results of these experiments are summarized in Fig. 3.

Behavior in steady-state conditions

In order to determine the relation of HiPIP-type Center S-3 to the location of the alternative oxidase in the higher plant mitochondria, the behavior of the center under a variety of steady-state conditions was examined. In these experiments both succinate and externally added NADH (which donates to the substrate side of coupling site II via a externally facing NADH dehydrogenase [19]) were used as respiratory substrates. The redox states of Center S-3 were examined in the presence of inhibitors of the main respiratory pathway (KCN) or the alternative respiratory pathway (salicylhydroxamic acid) or both. The results obtained with mitochondria from *Sy. foetidus* are presented in Table II This is a highly cyanide-insensitive tissue, the mitochondria typically exhibiting 50—80% insensitivity to 1 mM KCN. Addition of succinate (in the presence of ATP to activate the succinate dehydrogenase), with or without ADP caused a 30% reduction in Center S-3. The further addition of cyanide or salicylhydroxamic acid alone caused little or no further reduction of the center. However, when both cyanide and salicylhydroxamic acid were added together, a significant extra reduction of Center S-3 (to 65—85% reduced) occurred. Under anaerobic conditions with succinate as substrate, up to 90% of the center was reduced.

In *Sy. foetidus* mitochondria respiring with NADH as respiratory substrate, little reduction of the Center S-3 occurred (less than 15%) under aerobic conditions and in the absence of respiratory inhibitors. However, the addition of

TABLE II

THE REDOX STATE OF CENTER S-3 UNDER A VARIETY OF STEADY-STATE RESPIRATORY CONDITIONS

The redox state of Center S-3 was determined from the $g = 2.018$ EPR signal peak height while defining the no substrate (aerobic) spectrum as the fully oxidized condition. All spectra were recorded at 8°K and 5 mW of power. Details of the experimental procedures are presented in Materials and Methods.

Respiratory condition	Percent oxidized Center S-3 remaining *		
	Symplocarpus foetidus	*Sauromatum guttatum*	Potato tuber
Aerobic (control)	100	100	100
+ succinate/ATP	68	77	88
+ succinate/ATP/ADP	74	n.t.	104
+ succinate/ATP/KCN	57	96	65
+ succinate/ATP/salicylhydroxamic acid	68	42	93
+ succinate/ATP/salicylhydroxamic acid/KCN	25	41	45
+ succinate/ATP/anaerobic	20	<10	<20
+ NADH	89	66	108
+ NADH + KCN	74	57	118
+ NADH/salicylhydroxamic acid	62	29	95
+ NADH/KCN/salicylhydroxamic acid	47	14	95
+ NADH/anaerobic	24	<10	<20
Percent insensitivity of succinate oxidation to 1 mM KCN	50—80	70—100	<5

* Figures accurate to ±15% of aerobic (control) value.
n.t., not tested.

either KCN or salicylhydroxamic acid to these respiring mitochondria caused a significant further reduction of the center (to about 30% reduced), in contrast to the result obtained when succinate was the substrate. Addition of both KCN and salicylhydroxamic acid together caused greater than 50% reduction and anaerobiosis caused greater than 75% reduction of the center.

These results are interpreted as evidence that the alternative oxidase is on the high potential side of Center S-3, i.e. it tends to keep Center S-3 oxidized. In the case of *Sy. foetidus*, both the main and alternative respiratory pathway have about the same electron-transporting capacity and so the addition of cyanide or salicylhydroxamate alone in the presence of substrate had only a small effect on the redox state of Center S-3. Only when both were added together was a significant extra reduction seen. Also included in Table II are results obtained with the even more highly cyanide-insensitive mitochondria isolated from *Sa. guttatum* spadix. In this case it can be seen that salicylhydroxamic acid caused a significant reduction of Center S-3 with either NADH or succinate as substrate, even without the prior addition of KCN. This is presumably caused by the fact that the electron-transporting capacity of the alternative pathway is much greater than that of the main respiratory pathway in these mitochondria. This would also predict that cyanide addition should have little effect on the level of Center S-3 reduction in the presence of succinate or NADH, and this is in fact observed.

The results with the cyanide-sensitive mitochondria of potato tubers (Table

II) act as a control to these experiments in that addition of salicylhydroxamic acid to respiring mitochondria in the presence or absence of KCN does not significantly affect the redox state of the Center S-3.

The relation between cyanide-insensitive NADH oxidase activity and the presence of an active succinate dehydrogenase

Experiments were performed to determine whether a component of succinate dehydrogenase was necessary for cyanide-insensitive respiratory activity. Mitochondria isolated from the spadix of *Sa. guttatum* were used (respiratory activities were 70—100% insensitive to 1 mM KCN). The mitochondria, after suitable dilution in distilled water containing 1 mg/ml bovine serum albumin and 0.3 mM ATP, were French-pressed at 2000 lb/inch^2. The resulting suspension was centrifuged at 7000 × *g* for 10 min to remove unbroken mitochondria. Half of the supernatant, was then adjusted to pH 9.9 with 1 M KOH, whilst the other half was kept as a control at pH 6.9. After incubation for 20 min at 0°C, both were centrifuged separately at 150 000 × *g* for 30 min to precipitate the submitochondrial particles. The cyanide-insensitive NADH- and succinate-supported respiration in these particles was assayed and the results are presented in Table III. EPR spectra of the particles after dilution in oxygenated medium A were also taken so that an estimate of the concentration of Center S-3 might be made. The results show (Table III and Fig. 4) that the alkaline treatment removed most of the succinate oxidase activity (measured in the presence of added cytochrome *c* so that cytochrome *c* content was not rate limiting) and that more than 90% of the Center S-3 signal (and hence, by inference, more than 90% of the succinate dehydrogenase, compare, for example, refs. 20 and 21) from the submitochondrial particles. Furthermore, the remaining NADH oxidase activity of these alkaline-washed particles retained its cyanide resistance (see Table III) and this cyanide-resistant activity was far in excess of the total electron-transferring capacity of the small residual succinate oxidase

TABLE III

THE RESPIRATORY ACTIVITIES OF *SAUROMATUM GUTTATUM* MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Preparations of particles are described in the text. In all cases oxygen consumption was measured with a Clark-type oxygen electrode. Concentrations of reagents were: succinate, 10 mM (added with 0.3 mM ATP to activate the succinate dehydrogenase); NADH, 1 mM; KCN, 1 mM. The cyanide was always added before substrate so that maximal inhibition was obtained.

	Washed mitochondria	Submitochondrial particles	
		Washed at pH 6.9	Washed at pH 9.9
NADH oxidase *	451	757	293
NADH + KCN *	369	401	234
Percent cyanide insensitivity	82%	53%	80%
Succinate oxidase *	369	178	15
Succinate + KCN *	369	134	15
Percent cyanide insensitivity	100%	75%	—

* nmol O_2 consumed/min per mg protein at 23°C.

activity. The cyanide-insensitive NADH oxidase activity was highly sensitive to salicylhydroxamic acid.

A similar experiment was performed with mitochondria isolated from *Sy. foetidus* spadix. Again alkaline (pH 9.5)-treated submitochondrial particles displayed no detectable succinate oxidase and yet the residual NADH oxidase activity was greater than 60% insensitive to 1 mM KCN added before or after substrate.

Discussion

It is clear from the data presented that the major higher plant mitochondrial HiPIP is analogous to Center S-3 of mammalian systems in terms of its physical properties and its association with succinate dehydrogenase. The absence or low levels of the second reported mitochondrial HiPIP [5] from higher plant mitochondria has simplified the characterization of Center S-3 to some extent. The only significant difference of the plant Center S-3 from its mammalian counterpart that we were able to detect was its midpoint potential of +65 ± 10 mV which was fully pH independent betwwen pH 7.0 and 8.5. The mammalian Center S-3 has been reported to have a midpoint potential of +120 mV which exhibits pH-dependent behavior above a pH of 7, see ref. 2 (but compare to ref. 4, for the E_m at pH 7, in isolated mammalian preparations). However, Ohnishi (personal communication) has recently found that the midpoint potential of Center S-3 of beef heart submitochondrial particles is pH independent. These and our results indicate that the uptake of a proton is not associated with reduction of the center, i.e. Center S-3 is an electron, rather than a hydrogen atom, carrier.

The relation of Center S-3 to the alternative oxidation step

The results of the steady-state experiments indicate that the operation of the alternative respiratory oxidase tends to keep Center S-3 oxidized. For example, the addition of salicylhydroxamic acid to those mitochondria which were able to respire in the presence of succinate plus cyanide, i.e. via the alternative pathway, caused an extra reduction of Center S-3. This is an important observation with respect to the location of the alternative oxidase. For example, it is apparent that several types of mitochondria display cyanide-insensitive respiration with succinate, but not with external NADH, as substrate [22—24] and this would tend to suggest that the alternative oxidation step itself is most closely associated with the succinate dehydrogenase complex, as has, in fact, been suggested by Lyr and Schewe [25]. The work described here, however, argues strongly against this possibility. The fact that Center S-3 is kept oxidized by the alternative oxidase indicates that the oxygen-reducing step is on the electron donating side of Center S-3. Since this center is presumably the one which connects the succinate dehydrogenase flavin, plus Centers S-1 and S-2 to the main respiratory pathway [4,26,27], this effectively rules out an autoxidation of any of these centers to account for alternative respiratory oxidations. The experiments with salicylhydroxamic acid further indicate that Center S-3 is not itself the autoxidizable component, since if this were the case one would expect a significant perturbation of the Center S-3 iron environment by this

potent inhibitor of the alternative oxidase. The lack of a hydroxamic acid effect, or in some cases the absence, of the Ruzicka (bc-3) center rules out a direct role of this HiPIP in alternative oxidations also.

It should be noted that in some cases (for example, see the results of the steady-state levels of Center S-3 of mung bean mitochondria described in ref. 8), it appears that a large proportion of the Center S-3 is reduced only sluggishly or not at all under steady-state conditions, although upon anaerobiosis the majority is both succinate and NADH reducible. The meaning of this is unclear at present, but suggests that a major part of Center S-3 may not be physiologically active in several types of plant mitochondria.

Some deductions may also be made from the steady-state experiments concerning the relation between Center S-3 and the pathway of external NADH oxidation. We assume (ref. 28 and compare ref. 29), in contrast to the recent remarks of Solomos [30], that the pathway of external NADH oxidation involves at least part of the quinone pool. This is necessary to explain the observed P/O ratio of 2 with external NADH [29] and is borne out by the fact that external NADH can remove the split $g = 2.00$ ubisemiquinone signal [8]. In mitochondria of mung bean hypocotyls [8] and potato tubers (this report), Center S-3 was not noticeably reduced by NADH unless the suspension went anaerobic, and this suggests that Center S-3 is not directly involved in electron transport with external NADH as substrate and that electron transport to it from NADH is sluggish in these mitochondria. Even in *Sa. guttatum* and *Sy. foetidus* mitochondria, which have rather high rates of external NADH oxidation, little reduction of Center S-3 was observed unless total electron flux to oxygen was severely inhibited.

These notions, for the sake of clarity, are summarized in a general scheme illustrated in Fig. 5. This model is based upon Mitchell's protonmotive ubiquinone cycle [31,32] as modified for the higher plant system [28]. The full scheme, which is outside the scope of this report, is simplified so that it defines only the ordering of components and does not define the semiquinone or protonmotive features which are central to these ideas (see, for example, refs. 28, 31 and 32). In this scheme, substrates (as succinate via succinate dehydrogenase or as NADH via the externally located flavoprotein) donate electrons to ubiquinone species. These form the fully reduced quinone either by a two-step reduction [31] or by dismutation [33]. This reduced quinone may then donate reducing power protonmotively to the $b \cdot c_1$ complex (and hence to cytochrome oxidase) or non-protonmotively directly to the alternative oxygen-consuming step. Antimycin A inhibits the main respiratory pathway by blocking the flow of electrons from the cytochromes *b* back to ubiquinone. This causes the cytochromes *b* to become fully reduced and hence prevents the electron flow from QH_2 to cytochromes c_1/c, but it does not prevent the flow of electrons from QH_2 to the alternative oxygen-consuming step. In cases where external NADH cannot be oxidized via the alternative oxidase, one must postulate that the quinone pool with which external NADH interacts is not in electronic contact with the alternatively oxidizable quinone pool.

The nature of the alternative oxidation step

Since salicylhydroxamic acid does not, as detected by electron paramagnetic

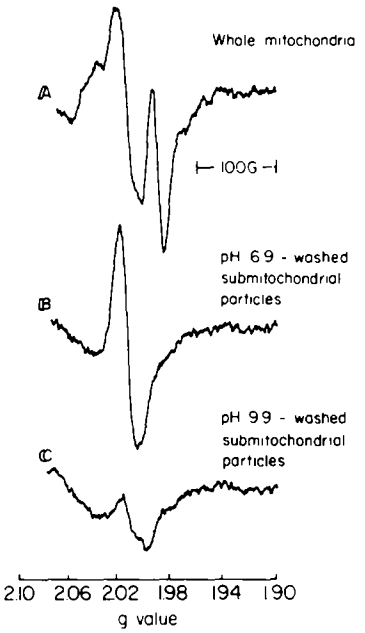

Fig. 4. First derivative EPR spectra of *Sa. guttatum* mitochondria and submitochondrial particles at 7.5°K. Samples were suspended in oxygenated medium A and frozen rapidly in liquid nitrogen (see Materials and Methods for preparation of samples). Protein concentrations were: washed mitochondria, 2.8 mg/ml; pH 6.9 washed submitochondrial particles, 2.7 mg/ml; pH 9.9 washed submitochondrial particles, 1.4 mg/ml. Other conditions of EPR measurements were: modulation amplitude, 12.5 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.19 GHz; receiver gain, $3.2 \cdot 10^3$; scan rate, 250 G/min; temperature, 7.5°K.

resonance, directly affect the HiPIP-type centers of plant mitochondria, it is highly unlikely that an iron-chelating effect on these iron sulfur centers is its mode of inhibition or that either of these centers is the site of alternative oxygen reduction. We have also found that the EPR signals of the ferredoxin-type centers S-1, S-2 and N-2 of mung bean mitochondria are not noticeably changed on addition of hydroxamic acids (Rich, P.R., unpublished data). These results are not wholly surprising since removal of iron from an intact iron sulfur center by chelation has not been demonstrated in any system within the time range of these experiments. If it did occur, it would require irreversible protein denaturation, a process which is unlikely in this case since inhibition of the alternative pathway by hydroxamic acids is reversible (unpublished data from this laboratory).

These considerations leave the conclusion that a component of the respiratory chain after succinate dehydrogenase is the autoxidizable component. We have already shown that hydroxamic acids are capable of perturbing the environment around the quinone pool which interacts with succinate dehydrogenase, although this effect requires a rather higher concentration of hydroxamic acid than is required to inhibit the alternative oxidation step [8]. It is unnecessary to consider the *b*-type cytochromes as candidates for the autoxidation since they are all fully reduced on addition of antimycin A [34] and, furthermore, the unusual cytochrome of spadix tissues, cytochrome b_7 [35,

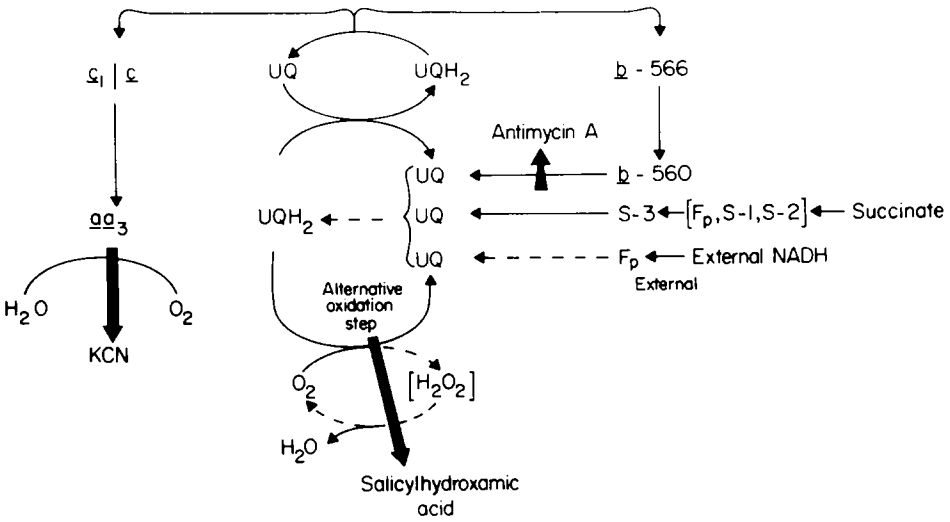

Fig. 5. Schematic representation of the possible organization of the higher plant electron transport chain. The scheme is based upon a protonmotive function of ubiquinone (represented by UQH_2 and UQ for its reduced and oxidized forms, respectively). Electrons from succinate dehydrogenase, external NADH dehydrogenase or cytochrome *b*-560 donate to ubiquinone which is located on the inner side of the inner membrane. The ubiquinones with which they interact may be separated either as different pools or redox states of ubiquinone. The reduced ubiquinone formed (either by a two-step reduction [31] or by dismutation [33]) may then be oxidized either by the alternative oxidation step or by a further quinone species located close to the $b \cdot c_1$ complex to which it donates electrons. The branchpoint of electron flow to the cytochrome oxidase pathway or to the alternative pathways is therefore at the level of a pool of ubiquinone. Fp, flavoprotein; S-1 and S-2, the ferredoxin-type centers of succinate dehydrogenase.

36], has been found to be reducible by succinate only sluggishly on anaerobiosis, and not reducible at all by malate or NADH under any conditions (Rich, P.R., unpublished data). Hence, we are left with the most likely candidate of a pool of quinone or an as yet EPR- and optically invisible component which interacts with this quinone pool as the autoxidizable component responsible for the cyanide-insensitive oxygen consumption. Studies are at present in progress to try to distinguish such a pool of specific quinone from the bulk, rather less specific, total quinone pool.

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References

1 Bartsch, R.G. (1963) in Bacterial Photosynthesis (Guest, H., San Pietro, A. and Vernon, L.P., eds.), pp. 315—326, Antioch Press, Yellow Springs
2 Ingledew, W.J. and Ohnishi, T. (1975) FEBS Lett. 54, 167—171
3 Beinert, H., Ackrell, B.A.C., Kearney, E.B. and Singer, T.P. (1975) Eur. J. Biochem. 54, 185—194
4 Ohnishi, T., Lim, J., Winter, D.B. and King, T.E. (1976) J. Biol. Chem. 251, 2105—2109

5 Ruzicka, F.J. and Beinert, H. (1974) Biochem. Biophys. Res. Commun. 58, 556—563
6 Ohnishi, T., Ingledew, W.J. and Shiraishi, S. (1976) Biochem. J. 153, 39—48
7 Moore, A.L., Rich, P.R., Bonner, Jr., W.D. and Ingledew, W.J. (1976) Biochem. Biophys. Res. Commun. 72, 1099—1107
8 Rich, P.R., Moore, A.L., Ingledew, W.J. and Bonner, Jr., W.D. (1977) Biochim. Biophys. Acta, 462, 501—514
9 Bendall, D.S. and Bonner, Jr., W.D. (1971) Plant Physiol. 47, 236—245
10 Bonner, Jr., W.D. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M E., eds.), Vol. X, pp. 126—133, Academic Press, New York
11 Wilson, S.B. and Bonner, Jr., W.D. (1970) Plant Physiol. 46, 25—30
12 Rich, P.R., Boveris, A., Bonner, Jr., W.D. and Moore, A.L. (1976) Biochem. Biophys. Res. Commun. 71, 695—703
13 Rich, P.R., Moore, A.L. and Bonner, Jr., W.D. (1977) Biochem. J. 162, 205—208
14 Ohnishi, T. (1975) Biochim. Biophys. Acta 387, 475—490
15 Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 395—407
16 Dutton, P.L. (1971) Biochim. Biophys. Acta 226, 63—80
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Schonbaum, G.S., Bonner, Jr., W.D., Storey, B.T. and Bahr, J.T. (1971) Plant Physiol. 47, 124—128
19 Douce, R., Mannella, C.A. and Bonner, Jr., W.D. (1973) Biochim. Biophys. Acta 292, 105—116
20 King, T.E. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 322—332, Academic Press, New York
21 Ingledew, W.J. and Prince, R.C. (1977) Arch. Biochem. Biophys. 178, 303—307
22 Hackett, D.P., Haas, D.W., Griffiths, S.K. and Niederpruem, D.J. (1960) Plant Physiol. 35, 8—19
23 Dizengremel, D. (1975) Physiol. Vég. 13, 39—54
24 van der Plas, L.H.W. (1977) Ph.D. Thesis, Free University, Amsterdam, the Netherlands
25 Lyr, H. and Schewe, T. (1975) Acta Biol. Med. Ger. 54, 1631—1641
26 Ohnishi, T., Salerno, J.C., Winter, D.B., Lim, J., Yu, L. and King, T.E. (1976) J. Biol. Chem. 251, 2094—2104
27 Ingledew, W.J., Salerno, J.C. and Ohnishi, T. (1976) Arch. Biochem. Biophys. 177, 176—184
28 Rich, P.R. and Moore, A.L. (1976) FEBS Lett. 65, 339—344
29 Palmer, J.M. (1974) Horizons Biochem. Biophys. 1, 220—260
30 Solomos, T. (1977) Annu. Rev. Plant Physiol. 28, 279—297
31 Mitchell, P. (1975) FEBS Lett. 59, 137—139.
32 Mitchell, P. (1976) J. Theor. Biol. 62, 327—367
33 Kroger, A. (1976) FEBS Lett. 65, 278—280
34 Lance, C. and Bonner, Jr., W.D. (1968) Plant Physiol. 43, 756—766
35 Bendall, D.S. and Hill, R. (1956) New Phytol. 55, 206—212
36 Bendall, D.S. (1958) Biochem. J. 70, 381—390