ON THE REACTIVITY OF OXYGEN WITH PHOTOSYSTEM I ELECTRON ACCEPTORS

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1. Introduction

The reduction of molecular oxygen as a consequence of photosynthetic electron transport was initially demonstrated [1,2] where oxygen was shown to serve as a Hill oxidant. Subsequently, it was recognized that the major site for reduction of oxygen is via photosystem I [3–5]. In the absence of an artificial electron donor system, oxygen reduction by illuminated broken chloroplasts (poisoned with KCN to inhibit catalase) was shown [5] to be saturated by an oxygen tension equivalent to 4% (partial pressure) oxygen in nitrogen. Further, an app. K_m of 5 μM was measured [5] for oxygen with the broken chloroplast preparation. Since soluble ferredoxin was assumed to be absent, the app. K_m was suggested [5] to reflect the affinity of the PSI reductant (other than ferredoxin) for molecular oxygen. There is now substantial evidence to indicate that the endogenous Mehler reaction (oxygen reduction to produce hydrogen peroxide) involves a univalent reduction of oxygen and formation of a superoxide free radical [6,7]. The photoreduction of cytochrome *c* mediated by both ferredoxin and by superoxide radical was studied [7]. Photoreduction of cytochrome *c*, under aerobic conditions and in the presence of 0.5–1 μM ferredoxin, was poorly inhibited (< 20%) by a saturating amount of superoxide dismutase. Thus, under their experimental conditions, the reduction of cytochrome *c* appeared to proceed mainly via direct electron transfer through ferredoxin rather than via an indirect reaction mediated by superoxide radical. That is, ~1 μM ferredoxin competed favorably and effectively with the large excess of oxygen* for electrons from the reducing end of PSI such that no significant amount of superoxide radical was produced. These findings imply that:

(1) The affinity of the PSI reductant for ferredoxin is very much greater than for molecular oxygen;
(2) Even in the presence of ~250 μM oxygen, reduced ferredoxin is preferentially oxidized by cytochrome *c* (present at 20 μM).

The formation of complexes of cytochrome *c* and ferredoxin in aqueous solutions which exist as stable physical entities separable by gel permeation chromatography has been shown [8]. The involvement of such complexes in the ferredoxin-mediated photoreduction of cytochrome *c* catalyzed by PSI provides a tempting explanation for the preferential channeling of electrons from the endogenous PSI reductant to cytochrome *c*. On the other hand, it is still rather difficult to understand the inefficient competition by 250 μM O_2 with 1 μM ferredoxin especially in view of the high affinity of the PSI reductant for oxygen (app. K_m 5 μM) reported [5].

We report here the effect of oxygen tension on PSI-catalyzed oxygen uptake by subchloroplast fragments in reactions involving:

(1) The endogenous PSI reductant;
(2) Externally added soluble ferredoxin;
(3) Addition of a low potential autooxidizable electron carrier, methyl viologen.

Abbreviations: DCPIP, dichlorophenolindophenol; chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; PSI, photosystem I; PSII, photosystem II

* Approximately 250 μM oxygen is present in an aqueous solution in equilibrium with air

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2. Materials and methods

To avoid kinetic complications due to a limiting rate of electron donation, an efficient PSI donor system consisting of 5 mM Na-ascorbate, 0.1 mM DCPIP and 10 μM spinach plastocyanin was employed in all assays. In addition, 5 μM DCMU was present in the reaction mixture to isolate PSI from the reductant generated by PSII. Sodium azide (1 mM) was included in all assays to inhibit catalase.

Oxygen tension (as % of air saturation) was measured with a membrane-covered, Clark-type oxygen electrode and displayed on the *X*-axis of an *X*–*Y* recorder. The time derivative of oxygen tension (that is, the rate of reaction) was obtained by analog electronic differentiation of the current signal from the Clark-type oxygen electrode and displayed on the *Y*-axis of the recorder [9].

Subchloroplast fragments were prepared from spinach chloroplasts [10] by:

(i) Washing in 1 mM EDTA (pH 8);
(ii) Sonication for 1.5 min at full power with a Branson Sonifier in a medium containing chloroplasts equivalent to 0.5 mg chl/ml and 50 mM Tricine–NaOH, (pH 7.8);
(iii) Centrifugation at 5000 × *g* for 20 min to remove large fragments;
(iv) Centrifugation of the supernatant solutions at 40 000 × *g* for 60 min to collect the subchloroplast fragments which were resuspended in 50 mM Tricine–NaOH (pH 7.8) supplemented with 0.25 M sucrose and 50 mM KCl.

The above operations were carried out at 0–4°C. Chlorophyll concentration was determined spectrophotometrically [11].

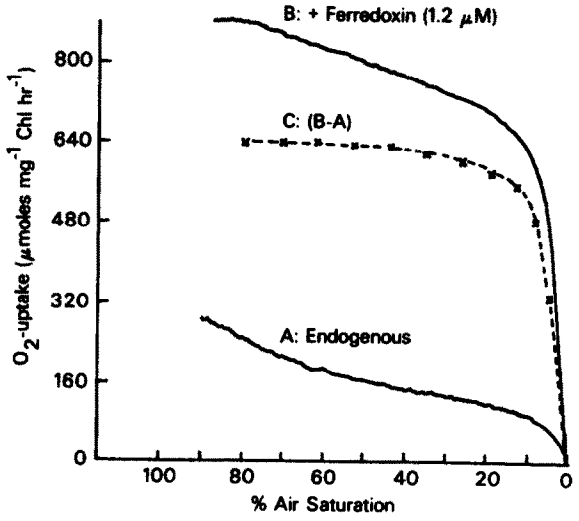

Fig.1. Effect of oxygen partial pressure and ferredoxin on rate of oxygen consumption by illuminated subchloroplast fragments. Subchloroplast fragments were suspended at a concentration equivalent to 12 μg chl/ml in a reaction mixture (2.0 ml total vol.) containing: 0.25 M sucrose; 50 mM Tricine–NaOH (pH 7.8); 50 mM KCl, 2 mM $MgCl_2$, 5 mM Na ascorbate; 0.1 mM DCPIP; 1 mM sodium azide; and 5×10^{-6} M DCMU. In darkness, the oxygen partial pressure was adjusted to 90–115% of air saturation at 25°C by slowly bubbling oxygen through the sample. After recording the low rate of light-independent oxygen uptake (due to slow auto-oxidation of reduced DCPIP), the endogenous light-dependent oxygen uptake (trace A) was initiated by illuminating the sample with a saturating intensity of white light (1×10^{-6} ergs s^{-1} cm^{-2}, excluding infrared). Where noted, 1.2 μM spinach ferredoxin was included (trace B). The stimulation by ferredoxin of oxygen uptake is indicated by curve C. Due to the lag in the response of the electrode differentiator system, the initial segments of traces A and B (> 95% and 78%, respectively) are highly distorted.

3. Results and discussion

The effect of oxygen partial pressure on the rate of PSI-catalyzed oxygen consumption by sonicated subchloroplast fragments is depicted in fig.1. The endogenous rate of oxygen uptake by illuminated subchloroplast fragments in the presence of an efficient PSI donor system showed a strong dependence on the oxygen partial pressure of the medium (fig.1, trace A). At ~100% air saturation, oxygen was taken up at 330 μmol. mg chl^{-1}. h^{-1}. The rate decreased to ~50% as the oxygen partial pressure of the sample was reduced to ~50% of air saturation. From 50–10% of air saturation, a much more gradual decrease in the rate of oxygen uptake occurred. When the oxygen partial pressure was reduced to < 10% of air saturation, a very drastic reduction in the rate of oxygen consumption was observed. Apparently, the reduction of molecular oxygen by illuminated subchloroplast fragments is not catalyzed by a single, homogeneous species of PSI reductant. It is more likely that endogenous oxygen reduction by the Mehler reaction involves a minimum of two autooxidizable, redox

species having drastically different affinities for molecular oxygen.

Upon addition of 1.2 μM soluble ferredoxin a much higher rate of oxygen uptake was obtained (fig.1, trace B). Interestingly, at oxygen partial pressures > 10–15% of air saturation, trace B nearly parallels trace A. That is, in absolute terms, the ferredoxin stimulation of the rate of oxygen uptake attending a given change of oxygen partial pressure is nearly constant above 10–15% of air saturation. These results are most readily understood if one assumes that the electron supply to PSI and its photochemistry are not rate limiting. Under these conditions, addition of ferredoxin induces an additional oxygen-consuming reaction which is superimposed on the endogenous reaction of the subchloroplast fragments. The dependence of the ferredoxin-mediated reaction on oxygen partial pressure is better visualized by subtracting trace A from trace B; the difference is plotted as trace C of fig.1. Clearly, reduced ferredoxin generated via photochemical reduction by PSI reacts efficiently with molecular oxygen.

One mole of clostridial ferredoxin was shown [12] to catalyze the formation of 65 mol superoxide/min when reacting with molecular oxygen at pH 7.8. In fig.1, 1.2 μM spinach ferredoxin induced a net increase of ~650 μmol oxygen. mg chl^{-1}. h^{-1} which corresponds to a net consumption of 128 nmol. oxygen. ml sample^{-1}. min^{-1}. That is, 1 mol ferredoxin reacts with 107 mol oxygen/min in a univalent reduction of molecular oxygen. Since spinach and clostridial ferredoxin react similarly in various photosynthetic electron transport reactions, it is not surprising that their rates of reaction with oxygen are comparable. It is also interesting to note that another nonheme iron–sulfur protein, milk xanthine oxidase, was reported to catalyze production of 242 mol superoxide. mol enzyme^{-1}. min^{-1} [12].

The rate of ferredoxin-stimulated oxygen consumption is saturated at rather low values of oxygen partial pressure. Further, the reaction reaches 50% of its maximal value at 3–5% of air saturation. Therefore, the app. K_m of reduced ferredoxin for oxygen should not be > 7.5–12.5 μM.

Using a similar assay system, we compared next the effect of oxygen partial pressure on the rate of PSI-dependent oxygen uptake:

(1) For the endogenous reaction;

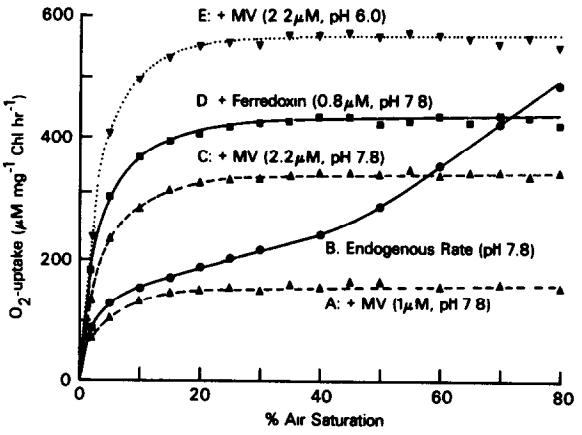

Fig.2. Oxygen saturation curves for the endogenous reaction and ferredoxin or methyl viologen stimulated reduction of oxygen. Basic reaction mixtures were identical to that of sample A in fig.1 except that: 1 μM methyl viologen was included for sample A; 2.2 μM methyl viologen for sample C; and 0.8 μM ferredoxin for sample D. Sample E is identical to sample C except that 50 mM MES–NaOH buffer (pH 6.0) replaced the Tricine–NaOH buffer (pH 7.8). Other assay conditions were identical to those in fig.1. Curve B was plotted from the original rates whereas curves A and C–E were plotted from the differential rates between the mediator-stimulated and endogenous reactions.

(2) For the reaction induced by addition of soluble ferredoxin;

(3) For the reaction stimulated by addition of an artificial, low potential, autooxidizable electron carrier, methyl viologen.

The results of these studies are summarized in fig.2. Clearly, both spinach ferredoxin and methyl viologen are effective catalysts for reduction of molecular oxygen. On an equimolar basis, the natural carrier, ferredoxin, is the better electron mediator between the PSI reductant and oxygen (compare curve D with curves A or C). That is, the PSI reductant has a higher affinity for its physiological acceptor, ferredoxin, than for methyl viologen. However, there appears to be very little difference in the affinity of reduced ferredoxin or methyl viologen for molecular oxygen. The oxygen-dependence curves of fig.2 demonstrate that both mediator-stimulated reactions are saturated at similar oxygen partial pressures. Further, both reactions exhibit 50% of their maximal rates at 3% of air saturation. Thus, the app. K_m for oxygen in both

cases must be very similar and should not be $> 7.5\ \mu M$.

In these studies, the 'on-going' rate rather than the initial rate of reaction was measured and plotted against the oxygen partial pressure of the medium. Unless all products are disposed of irreversibly, the value of the 'on-going' rate of oxygen consumption will depart progressively from the actual value of the 'initial rate' as the concentration of oxygen in the sample decreases. Furthermore, the response time of the Clark-type oxygen electrode increases as the oxygen content of the medium approaches zero. Therefore, the value of the app. K_m measured under the limiting lower range of oxygen partial pressures may simply reflect a severe distortion imposed by an inadequate electrode response time. For these two reasons, the app. K_m 7.5 μM reported here must be regarded as an upper limiting value.

As mentioned earlier, the endogenous reaction of subchloroplast fragments appears to be mediated by two autooxidizable redox agents having different affinities for molecular oxygen (see fig.1, trace A; fig.2, trace B). Based on the shape of the curves (fig.2), it appears that the app. K_m of the high oxygen-affinity redox species is very close to that of soluble ferredoxin and methyl viologen. Whether or not the high oxygen-affinity redox species represents a small amount of soluble ferredoxin which becomes tightly bound to the membrane fragments or represents actually one of the native membrane-bound electron acceptors of PSI requires further clarification. Using a monospecific antiferredoxin immunoglobulin, evidence was obtained [13] suggesting that a small quantity of soluble ferredoxin (bound to the thylakoid membranes) is responsible for the formation of ATP coupled to the endogenous pseudo cyclic electron flow from water to oxygen. However, the presence of the 'membrane-bound', immunological equivalent of soluble ferredoxin could not be shown in sonicated thylakoid membrane preparations [14]. We have encountered significant variation in the rate of endogenous oxygen uptake which was assigned to the high oxygen-affinity redox agent. Preliminary experiments indicated that prolonged sonication followed by sucrose density centrifugation and Sephadex G-75 chromatography resulted in a subchloroplast preparation which exhibited a lower rate of endogenous oxygen uptake at low partial pressure of oxygen (fig.3). The implications of this finding require further studies.

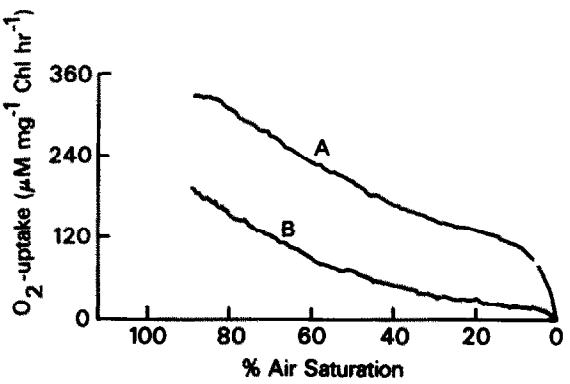

Fig.3. Endogenous oxygen uptake with two types of subchloroplast preparations. Assay conditions and reaction mixtures were as in fig.1. No redox mediators were added to the samples. Trace A was obtained with a subchloroplast preparation equivalent to that used in fig.1 and 2. Trace B was obtained with a different type of subchloroplast preparation isolated as follows: 80 ml EDTA washed spinach chloroplasts (precipitate III in [15]) were suspended in a medium containing 1 mM EDTA and 25 mM MES–NaOH (pH 6.0) at 0.3 mg chl/ml and sonicated in the cold (4°C) for 4 min total with a Branson Sonifier operated at full power in 40 s bursts separated by 30 s intervals. The sonicated samples were layered on top of a sucrose containing medium (15% sucrose by vol. 1 mM EDTA, 25 mM Tricine–NaOH (pH 7.8) and 25 mM KCl) and centrifuged at 26 000 rev./min in a Beckmann SW27 rotor for 6 h. A sharp green band located at the interface between the 15% sucrose medium and heavier medium containing 60% sucrose by vol., 25 mM Tricine–NaOH and 25 mM KCl was withdrawn and dialyzed against 100 vol. 50 mM Tricine–NaOH (pH 7.8) containing 10 mM KCl. Dialyzed samples were applied to a column of Sephadex G-75 (50 ml bed vol./ml sample) equilibrated and eluted with the same buffer. The peak green fractions which eluted at the void volume were pooled and used in the assay.

Acknowledgements

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