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"AUTOREDUCTION"—AN UNUSUAL PROPERTY OF PURE SPINACH CYTOCHROME *f*

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SUMMARY

Pure spinach cytochrome *f* exhibits the anomalous property of autoreduction at physiological pH. After complete oxidation followed by removal of oxidant, about 80% of the cytochrome *f* content appeared in reduced form without addition of exogenous reductant. The phenomenon was observed with two different buffers, two different oxidants, and three different methods of removing the oxidant.

While working with pure spinach cytochrome *f*, we observed that, upon removing excess oxidant from a fully oxidized solution of cytochrome *f*, this hemoprotein spontaneously became almost fully re-reduced. Accordingly, the term "autoreduction" (for lack of a better term) is defined experimentally in this paper as spontaneous re-reduction of pure cytochrome *f* at pH 8, 0 to 5 °C, without any addition of exogenous reducing agent and/or catalyst upon removal of excess oxidant. Autoxidation (direct oxidation of reduced redox proteins by oxygen) is not rare either *in vitro* or *in vivo*. In contrast, "autoreduction" of a pure protein is anomalous. (Recently[1] autoreduction of pure cytochrome *c* was observed to occur very slowly *in vitro* under unusual experimental conditions of pH. Since the experimental conditions used for autoreduction of cytochrome *c* differed markedly from the present studies, and since the molecular properties of cytochromes *c* and *f* also differ, comparison of the two phenomena does not appear feasible at present.) A gradual autoreduction was reported for impure plastocyanin but the purified protein did not exhibit this phenomenon[2].

The studies reported here were designed to examine more fully and establish this unusual phenomenon of "autoreduction" of cytochrome *f*. (These studies were presented previously at a meeting (June, 1971, Toronto) of the Canadian Biochemical Society.)

The following studies were performed without special efforts to exclude light or oxygen from the solutions. Table I summarizes the experiments performed to observe "autoreduction" of cytochrome *f*. Fully reduced pure cytochrome *f*[3] in 0.05 M Tris–HCl, pH 8, was completely oxidized by the addition of 0.001 M potassium ferricyanide at 0° C (incubation time of about 10 min). Spectrophotometric examination confirmed that cytochrome *f* was completely oxidized in that the characteristic α-peak of the reduced cytochrome had disappeared and been replaced by the oxidized spectrum of cytochrome *f*. (The absorption of ferricyanide prevents reliable detection

of the Soret peak of oxidized cytochrome *f*, but with persulfate (colorless) as oxidant, *vide infra*, the oxidized Soret was indeed observed as a secondary check on complete oxidation.) The presence of excess amounts of the oxidant, potassium ferricyanide, was indicated by the characteristic yellow color of the solution. Excess ferricyanide was then removed by percolation through a Sephadex G-25 desalting column equilibrated with 0.05 M Tris–HCl, pH 8. The cytochrome was excluded, eluted early and without overlap from the retarded ferricyanide fraction. 70–80 % of the original cytochrome content was recovered in the reduced form despite the fact that no external reducing agent had been added. This experiment was repeated several times with the same results using concentrations of cytochrome *f* ranging in absorbance from 0.05 to 0.15 ($\varepsilon_{mM} = 29.0$; 2.1 mg/ml = 1 absorbance unit, calculated from ref. 3). The Phoenix–Chance spectrophotometer used had a detection limit of 0.0002 absorbance unit (1 cm cell). In all cases subsequent addition of sodium dithionite (a strong reducing agent) to the eluted cytochrome did not significantly increase the amount of cytochrome in the reduced form. This indicated that very little of the cytochrome was recovered in the oxidized form and that virtually all of the recovered cytochrome was in the reduced form before attempts at chemical reduction. The lack of recovery of 100 % of the original cytochrome is attributable either partly to normal mechanical losses during the removal of oxidant or possibly to some irreversible inactivation of cytochrome *f* either during the stage of chemical oxidation with ferricyanide or during "autoreduction".

TABLE I

"AUTOREDUCTION" PHENOMENON FOR PURE CYTOCHROME *f* (*in vitro*)

I. 0.05 M Tris–HCl, pH 8; 0 °C.

One cycle:	*100% reduced cytochrome f*	*0.001 M ferricyanide* →	*100% oxidized cytochrome f + excess oxidant*	*removal of oxidant* →	*% re-reduced cytochrome f (no reductant added)*
		Oxidant		*Method used to remove oxidant*	
		(a) Ferricyanide		(a) Sephadex G-25	70–80
				(b) Dialysis	70–80
				(c) Electrophoresis	—*
		(b) Persulfate		(a) Sephadex G-25	70–80

I′. One cycle, but cytochrome was left 24 h (0 °C) in ferricyanide solution (pH 8). Result as above (increased time in oxidant didn't decrease amount of "autoreduction").

II. 0.05 M phosphate, pH 7.5.
All conditions above were repeated; all results were the same. Therefore, $-NH_2$ of Tris was not the reductant.

III. Two cycles: about 40–60% re-reduced cytochrome *f*.

* Qualitative spectrophotometric analyses (400–620 nm) of cytochrome *f* in gel slices showed that the pink band in the gel exhibited a strong reduced spectrum of cytochrome *f*. The Soret peak (410 nm) of oxidized cytochrome *f* was not detected in any gel slice either as a distinct peak or as an asymmetry in the spectrum of autoreduced cytochrome *f*.

The E_0' value for ferricyanide (0.36 V[4]) is quite close to the value of 0.365 V proposed for cytochrome *f* by Davenport and Hill[5]. Although complete oxidation of the heme group of cytochrome *f* was achieved in our experiments with excess ferricyanide, it is conceivable that some ferrocyanide ions (colorless) might have remained bound to cytochrome *f*, even after percolation through a Sephadex column, and that bound ferrocyanide ions could have subsequently re-reduced the cytochrome. Accordingly, the experiment was repeated replacing ferricyanide by ammonium persulfate (colorless), a much stronger oxidant (E_0 for $S_2O_8^{2-}$, SO_4^{2-} = 2.0 V *vs* 0.36 V for $Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$ (ref. 6)). Nevertheless, the results were the same (*cf.* Table I).

To eliminate the possibility, however minor, that the cytochrome was artifactually reduced during the stage of removal of ferricyanide by percolation through a Sephadex G-25 column, alternative procedures—dialysis or disc electrophoresis—were employed for removal of the ferricyanide oxidant as part of the "autoreduction" experiments. Table I shows, however, that as with Sephadex G-25, about 80 % "autoreduction" occurred after removing the oxidant by extensive dialysis against 0.05 M Tris–HCl, pH 8. Analytical disc gel electrophoresis was performed by the standard system of Davis[7] (*cf.* ref. 3) with a 4 % stacking gel and 5 % resolving gel. The sample, containing excess ferricyanide, 0.05 M Tris–HCl, pH 8, was applied after supplementation to 10 % concentration in sucrose. Within 15 min of applying the voltage, a yellow band (ferricyanide) moved rapidly away from the sample and the characteristic pink (reduced) cytochrome band could be seen in the gel. Upon slicing and analyzing the gel spectrophotometrically (without added reducing agent), it was found that the cytochrome had indeed become reduced.

To eliminate the small possibility that amino groups of Tris–HCl buffer could have re-reduced cytochrome *f* (*via* a possible amine oxidase reaction) the "autoreduction" experiment was performed again but with 0.05 M potassium phosphate buffer, pH 7.5 replacing Tris–HCl. The result was the same.

Complete oxidation of the heme of cytochrome *f* is observed immediately upon addition of oxidant, *i.e.* without any obvious lag, and, in any event, exposure of cytochrome *f* to excess ferricyanide or persulfate ions lasted about 20 to 30 min (pH 8, 0 °C). This period included 5–10 min of incubation with oxidant and about 20 min during the Sephadex column procedure before cytochrome *f* and oxidant were separated on the column. However, if non-heme redox groups of cytochrome *f* were responsible for its re-reduction, they might not be as quickly accessible to ferricyanide oxidation. Accordingly we examined the effect of leaving cytochrome *f* in excess ferricyanide solution (pH 8, 0 °C) for 24 h, after which ferricyanide was removed. The result was unchanged: no decrease in the amount of "autoreduction" was observed.

On performing two cycles of "autoreduction", *i.e.* taking the re-reduced cytochrome after one cycle, reoxidizing it and again removing the oxidant, 40–60 % of the original cytochrome was recovered in reduced form. Again no increase in the amount of reduced cytochrome was observed on adding sodium hydrosulfite (dithionite) to the recovered cytochrome.

The pure, dialyzed cytochrome *f* samples used in these studies contained no protein contaminants but probably contained some residual bound Triton X-100 (which had been used in the early purification steps (*cf.* ref. 3). However, Triton is an unlikely reductant of cytochrome *f* for two reasons: (1) The addition of 0.1 %

Triton X-100 to reduced cytochrome *f* in 0.05 M Tris–HCl, pH 8 (containing no dithiothreitol or other reducing agent) results both in disaggregation and in a slow autoxidation until only about 50 % of the initial reduced spectral intensity remains. (The fully reduced spectrum is largely restored by ascorbate.) (2) No other component, in our experience, is reduced by Triton X-100 (or Tween 80), *e.g.* both chloroplast cytochrome b_{559}[8] and cytochrome b_6 (unpublished) are in the oxidized form unless an exogenous reductant is added.

Determination of the half-time or other kinetic information concerning "autoreduction" has been precluded thus far by the relatively long time needed for removal of oxidant. Even with the quickest method (15 min *via* disc gel electrophoresis) the characteristic pink color of the reduced cytochrome reappeared without obvious time-lag once the dominant yellow ferricyanide band had moved ahead.

The previous experiments have demonstrated that "autoreduction" of pure cytochrome *f* occurs, but the source of electrons remains unidentified. A simple hypothesis (among many possibilities) would be the existence in cytochrome *f* of a low-potential reducing site electrically accessible to the known *c*-type heme group[3,5]. If so, the experiments presented require that electron-transfer between the two sites be hindered when exogenous oxidant is present since, otherwise, oxidation of the low-potential site would occur indirectly *via* oxidation of the heme groups, which we have found to be completely available to excess oxidant. The low-potential site would also be sterically shielded from direct oxidation by added oxidant. Since a second cycle of "autoreduction" was demonstrated, either there is more than one low-potential group per heme group or a mechanism exists for a reductive endergonic regeneration of low-potential site(s). Pure cytochrome *f* contains no non-heme iron, and flavin chromophores are not observed in cytochrome *f*'s absorption spectra[3]. A low-potential sulfhydryl group (perhaps cysteines within the protein moiety) is but one of many possibilities for the identity of the hypothetical low-potential site(s). Alternative or additional factors in generation of reducing equivalents might be the participation of light or conformational changes in the molecule.

"Autoreduction" of cytochrome *f* might be relevant in three ways: (1) Potentially, "autoreduction" could have great physiological importance for photosynthetic electron transfer if it can be shown at least *in vitro* that it occurs quickly enough. A more rapid removal of excess oxidant poses a technical precondition at present to kinetic studies. In addition, since cytochrome *f in vivo* is tightly bound and possibly restrained in disaggregated form (*cf.* ref. 3, p. 3541) by other chloroplast entities, demonstration of "autoreduction" of the cytochrome's form *in vivo* would be required. (2) The redox potential of cytochrome *f* (0.365 V, Davenport and Hill[5]) was determined using ferricyanide with the implicit assumption of only one redox site, that of the heme group. If a second, low-potential site is completely unavailable to both heme and ferricyanide when ferricyanide is present, the accepted redox value may well be validated but should nevertheless be re-examined. (3) The phenomenon, whatever its origin, is anomalous and interesting in its own right for physical and chemical study.

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