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Protochlorophyllide reductase: a flavoprotein?

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Protochlorophyllide reductase (EC 1.6.99.1) catalyses the light-dependent reduction of protochlorophyllide to chlorophyllide. Evidence has been obtained for the possible involvement of FAD in this reaction: (i) protochlorophyllide reductase is inhibited by quinacrine and trifluoperazine at micromolar levels consistent with their action as flavoprotein antagonists; (ii) preparations containing protochlorophyllide reductase can mediate cytochrome *c* reduction; (iii) in protochlorophyllide reductase-rich etioplast membranes FAD co-purifies with the enzyme.

Chlorophyll synthesis; Protochlorophyllide reduction; Flavoprotein

1. INTRODUCTION

The reduction of protochlorophyllide to chlorophyllide in higher plants is catalysed by the enzyme PCR which is found as a component of the plastid membranes [1]. The enzymology of PCR has attracted much interest undoubtedly due to the requirement for light in catalysis [1]. This enzyme is spectroscopically assayable in vitro by measuring photoinduced chlide formation in the presence of the two substrates, pchlide and NADPH [2]. Mechanistically it has been proposed that there are two steps in the overall reaction of PCR: firstly, the light-independent formation of a photoactive enzyme-pchlide-NADPH ternary complex; followed by the photoinduced hydrogen transfer from NADPH to pchlide [2]. In this paper we have used the in vitro PCR assay system to investigate the possibility of flavin involvement in the reaction.

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Abbreviations: PCR, protochlorophyllide reductase; pchlide, protochlorophyllide; chlide, chlorophyllide; EM, etioplast membrane (wheat); SGM, etioplast membranes purified on a sucrose gradient

2. MATERIALS AND METHODS

2.1. *Materials*

FAD, quinacrine, trifluoperazine and cytochrome *c* (horse heart) were purchased from Sigma. Wheat (*Triticum aestivum* Var. Avalon) was purchased from British Seed Houses, Avonmouth, England.

2.2. *Methods*

2.2.1. Membrane isolation

Etioplast membranes were prepared from 7-day-old etiolated wheat as previously described [3]. A PCR-enriched membrane fraction (SGM) was obtained by further purifying EMs on a sucrose gradient by the method of Beer and Griffiths [4]. The protein content of membranes was assayed by the following method of Bramhall et al. [5] and using bovine serum albumin as a standard.

2.2.2. Spectroscopy

Spectra of membranes resuspended in buffer [4] were obtained using a computer-linked sensitive split-beam spectrophotometer [6]. For the data in fig.2, EMs were scanned at time intervals against a blank of diluted milk; all spectra were subtracted from that recorded at time = 0 min to allow quantitation of the absorbance changes at 550 nm. 1 mM KCN was present in the EMs incubated with cytochrome *c* to inhibit any cytochrome oxidase activity arising from any small amount of contamination by mitochondrial membranes.

Fluorescence measurements were made with a FOCI mark 1 spectrofluorimeter. Flavins were extracted from SGMs by an adaptation of the method according to Faeder and Siegal [7]: SGMs were suspended in an extraction buffer (0.1 M KH_2PO_4, 0.1 mM EDTA, pH 7.7) to a final concentration of about 1 mg/ml. 1 ml samples were boiled for 3 min, cooled on ice and immediately quenched with an equal volume of 20% (w/v)

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trichloroacetic acid. Precipitated protein was removed by centrifugation (20000 × g, 30 min) and the supernatant adjusted to pH 7.7 with 300 μl of 0.5 M KOH prior to fluorimetry.

3. RESULTS AND DISCUSSION

Addition of quinacrine and trifluoperazine to standard PCR assays [2] gave linear but reduced rates of pchlide reduction. Fig.1 shows that both inhibitors were effective at micromolar concentrations with quinacrine being slightly more potent than trifluoperazine. In neither case did addition of further pchlide or NADPH alter the level of inhibition. Although trifluoperazine and quinacrine are known to inhibit a variety of protein functions [8–10] the inhibition at this low level is consistent only with their action as flavoprotein antagonists [11,12]. These data therefore suggest that a flavin might be involved in PCR catalysis.

Many flavin-linked enzymes possess diaphorase activity in which the reduced flavin generated during enzyme catalysis can reduce any one of a range of different artificial electron acceptors [13]. Therefore the possibility that PCR itself displays diaphorase activity was investigated. Fig.2 shows the time course of the reduction of the electron acceptor cytochrome *c* catalysed by EMs in the absence of any added reductant. Cytochrome *c*

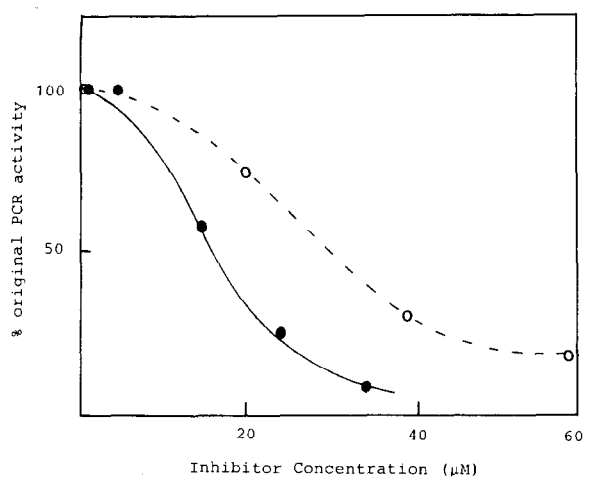

Fig.1. The effect of quinacrine (——) and trifluoroperazine (– – –) on protochlorophyllide reductase activity. Wheat etioplast membranes were assayed for PCR activity by the standard procedure [2] with inhibitors quinacrine (——) and trifluoroperazine (– – –) added to the reaction mixtures as indicated.

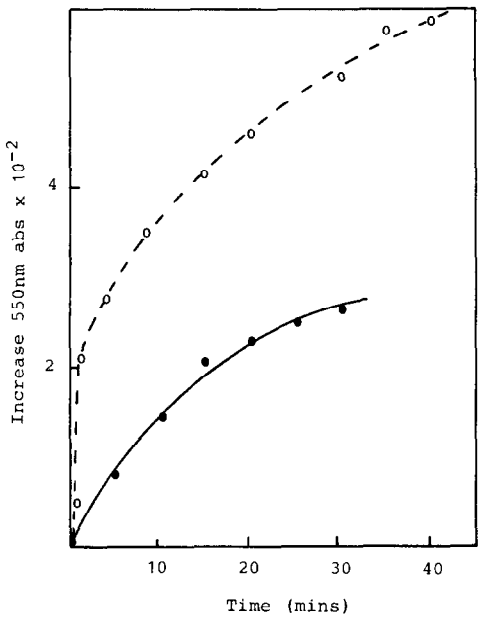

Fig.2. The reduction of cytochrome *c* by unilluminated (– – –) and illuminated (——) wheat etioplast membranes. Cytochrome *c* reduction was carried out spectrophotometrically as described in the text using wheat etioplast membranes (1.0 mg protein·ml^{-1}) with cytochrome *c* added at 0.5 mg·ml^{-1}. The illuminated sample was exposed to a focussed 55 W quartz-hydrogen lamp from a distance of 10 cm for 2 min.

was reduced rapidly by such preparations for the first 2.5 min and then at a much slower rate for at least a further 40 min. It will be recalled that such preparations have the PCR enzyme present as the ternary complex with reductant (NADPH) and pigment.

PCR is by far the major protein (approx. 40%) of these etioplast membranes. Its contribution to the observed diaphorase activity was assessed by repeating the cytochrome *c* reductase assay but using briefly illuminated (2 min white light from a focussed 12 V/55 W, quartz/halogen lamp at a distance of 25 cm) membranes. In such membranes, due to turnover of the PCR ternary complex, light would specifically deplete the reductase-bound NADPH such that in the absence of any added reductant the enzyme should now be unable to contribute to any of the diaphorase activity measured as cytochrome *c* reduction.

In the illuminated EMs the kinetics of cytochrome *c* reduction were clearly different with no initial rapid phase (fig.2) and only a slower rate

of reduction being present. This suggests that the NADPH in the PCR ternary complex is indeed the major source of reductant for the rapid phase of cytochrome *c* reduction observed in unilluminated membranes. Such a result, of course, implies that PCR itself is acting as a diaphorase – an activity considered to be a property of flavoenzymes [13].

Etioplast membranes can be enriched in PCR by sonication and fractionation on a sucrose gradient; in these purified membranes about 80% of the total membrane protein is PCR [4], and such preparations probably represent the purest source of PCR that can be prepared in sufficient quantities necessary for flavin analyses.

The fluorescence excitation spectrum of the flavin extract prepared from such membranes is shown in fig.3 (– – –). The figure also includes the corresponding spectrum of pure FAD (——). The similarity between the two spectra is quite marked, both showing multiple excitation maxima (525 nm emission) at 385, 468 and 450 nm. Although it is possible that the source of this FAD in the membrane is a protein other than PCR, the high (micromolar) levels of flavin in the extract strongly suggest that it can only be derived from the major SGM protein, namely PCR. Furthermore, in support of this proposal, previous experiments with the cruder EMs have already demonstrated a correlation between the capacity for light-induced chlide synthesis and endogenous concentration of extractable FAD, again indicating that the FAD may be derived from PCR [15].

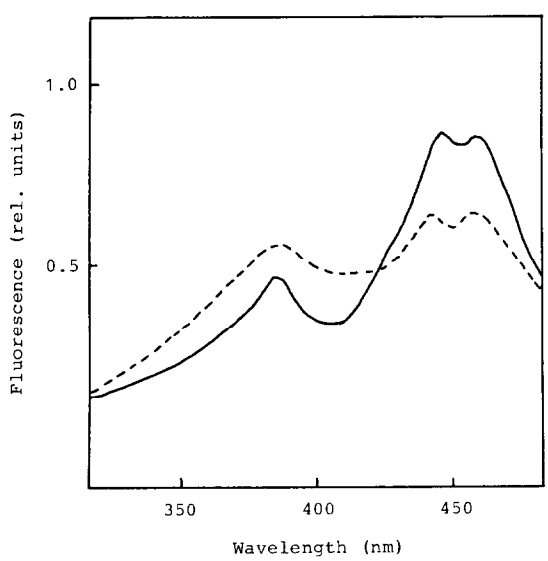

Fig.3. Fluorescence excitation spectra of an extract prepared from purified wheat etioplast membranes. Fluorescence emission was monitored at 525 nm. The extracts from the SGMs (– – –) were prepared as described in the text. The figure includes the excitation spectrum of FAD (——) for comparison.

In conclusion, our data show that PCR possesses some properties which can be considered characteristic of a flavin-linked enzyme. PCR is inhibited by both quinacrine and trifluoperazine at micromolar levels and is apparently able to mediate cytochrome *c* reduction. As would be expected, FAD is extractable from PCR-enriched SGMs; the high concentrations of FAD extracted and the predominance of PCR in the SGMs favours the source of FAD as being PCR itself. The well documented ability of flavins to mediate photoinduced redox reactions tends to strengthen further this suggestion.

An unambiguous identification of this postulated PCR cofactor must await purification of this enzyme in sufficient quantities for a more quantitative flavin analysis. Due to the lability of PCR, none of the methods so far published for its purification have proved practical for large scale preparation [4,16]. Recently, we have published an affinity chromatographic technique for PCR purification [17] and preliminary experiments have indicated that the eluted 'pure' fraction still retains an extremely high cytochrome *c* diaphorase activity. It is also possible that molecular biological techniques will provide further information on the nature of the PCR cofactors. In our lab we are currently sequencing PCR clones and have identified a possible FAD-binding site (Darrah, P., Teakle, G. and Griffiths, W.T., in preparation).

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REFERENCES

[1] Castelfranco, P.A. and Beale, S.I. (1983) Annu. Rev. Plant Physiol. 34, 241–278.
[2] Griffiths, W.T. (1978) Biochem. J. 174, 681–692.
[3] Griffiths, W.T. (1975) Biochem. J. 152, 623–635.
[4] Beer, N.S. and Griffiths, W.T. (1981) Biochem. J. 195, 83–92.
[5] Bramhall, S., Noak, N., Wu, M. and Loewenberg, J.R. (1969) Anal. Biochem. 31, 146–148.

[6] Parkinson, J.F. (1985) PhD Thesis, University of Bristol.
[7] Faeder, E.J. and Siegal, L.M. (1973) Anal. Biochem. 53, 332–336.
[8] Hunter, F.E.W. (1955) Methods Enzymol. 2, 611–616.
[9] Izawa, S. and Good, N.E. (1972) Methods Enzymol. 14, 355–375.
[10] Weiss, B. and Wallace, T.L. (1980) in: Calcium and Cell Function (Cheung, W.Y. ed.) vol.1, pp.330–367, Academic Press, London.
[11] Ernster, L. (1967) Methods Enzymol. 10, 309–317.
[12] Bellavite, P.S., Serra, M.C., Davoli, A., Bannister, J.V. and Rossi, F. (1983) Mol. Cell. Biochem. 52, 17–25.
[13] Edwards, Y.H., Potter, J. and Hopkinson, D.A. (1980) Biochem. J. 187, 429–436.
[14] Griffiths, W.T. (1974) FEBS Lett. 46, 301–304.
[15] Griffiths, W.T. and Walker, C.J. (1987) in: Progress in Photosynthesis Research (Biggins, J. ed.) pp.65–71, Balban International Sciences Services, Philadelphia.
[16] Apel, K., Santel, H.J., Redlinger, T.E. and Falk, H.R. (1980) Eur. J. Biochem. 111, 251–258.
[17] Richards, W.R., Walker, C.J. and Griffiths, W.T. (1987) Photosynthetica 21, 462–471.