Ultrafast catalytic processes and conformational changes in the light-driven enzyme protochlorophyllide oxidoreductase (POR)

Olga A. Sytina*, Derren J. Heyes†, C. Neil Hunter‡ and Marie Louise Groot*[1]

*Department of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands, †Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester M1 7DN, U.K., and ‡Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, U.K.

Abstract

The enzyme POR (protochlorophyllide oxidoreductase), from the family of alcohol dehydrogenases, reduces protochlorophyllide into chlorophyllide on the absorption of light. The reduction involves the transfer of two protons and two electrons and is an important regulatory step in the biosynthesis of chlorophyll. In recent years, due to the availability of large quantities of the pure enzyme, much of the catalytic reaction has been unravelled by using a variety of spectroscopic methods, including ultrafast initial events in catalysis. In addition, it has been demonstrated that a light-activated conformational change of the protein is necessary to activate catalysis. This makes POR a very important model system to study the relationship between structural changes of enzymes and functionality.

Introduction

The role of conformational changes in explaining the huge catalytic power of enzymes is currently one of the most challenging questions in biology [1–7]. Structural flexibility of proteins is considered to play a central role in, for example, the interaction between proteins, the interaction between proteins and substrates, allostery and catalytic activity [1–15]. Several enzymes have been reported to have a resting configuration and an active configuration, and the dynamic interconversion between such different conformational states is important for catalysis [13–15].

However, in principle, it is only possible to simultaneously study the real-time dynamics of catalytic intermediates and of conformational changes in enzymes where the reaction can be initiated by a trigger that is more rapid than the fastest dynamics involved. This possibility is afforded in light-driven enzymes, such as DNA photolyase, POR (protochlorophyllide oxidoreductase) and photosynthetic reaction centres. Reaction centres are transmembrane pigment–protein complexes that are responsible for catalysing light-driven charge separation and represent one of only a very few systems where electron transfer between redox centres can be monitored with femtosecond time resolution. The initial steps in the electron transfer reaction take place on a picosecond timescale, leading eventually to two-electron reduction of a bound quinone molecule on a millisecond timescale, after the subsequent absorption of two photons [16–18]. DNA photolyase induces the repair of UV-induced lesions in DNA by scission of covalent bonds between neighbouring pyrimidines. Photoactivation of an FAD cofactor occurs via a fast (picosecond timescale) multistep electron transfer through a chain of three tryptophan residues [19,20].

These examples demonstrate that light-driven enzymes provide the opportunity to study the initial ultrafast steps in catalysis. However, in the present review, we focus on another enzyme, POR, which requires light for both catalysis and to induce a conformational change, which switches the enzyme from an inactive into a fully active state. A detailed review on POR, including studies on the POR A and B isoforms from green plants, can be found in [21–23]. In the present study, we provide a limited overview of previous spectroscopic investigations of the isolated POR complex from cyanobacteria, with a particular focus on recent time-resolved transient absorption experiments.

POR

POR catalyses the *trans* addition of hydrogen from NADPH across the C-17–C-18 double bond of the D-ring of Pchlide (protochlorophyllide) to produce Chlide (chlorophyllide) [24]. This is an important regulatory step in chlorophyll biosynthesis and the subsequent assembly of the photosynthetic apparatus [23–25]. Catalysis is induced by the absorption of light by the substrate Pchlide and, as the enzyme–substrate complex can be pre-formed in the dark, this allows the reaction to be triggered by a short laser pulse to follow the dynamics without the interference from substrate and cofactor binding reactions [23]. It is proposed that a conserved tyrosine residue donates a proton to the C-18 position [26] and a hydride is transferred from the pro-S face of NADPH to the C-17 position of the Pchlide molecule

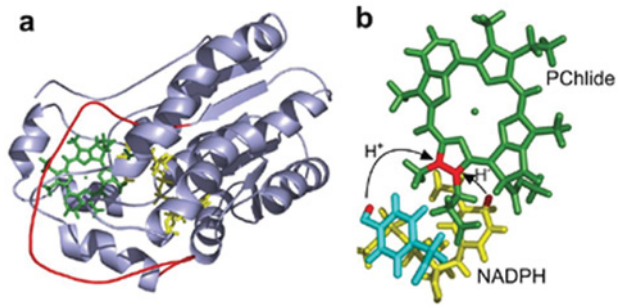
Key words: chlorophyllide, conformational change, Fourier-transform infrared (FTIR), hydride, protochlorophyllide oxidoreductase (POR).

Abbreviations used: Chlide, chlorophyllide; Pchlide, protochlorophyllide; POR, protochlorophyllide oxidoreductase.

[1]To whom correspondence should be addressed (email ml.groot@few.vu.nl).

Figure 1 | Homology model of POR from *Synechocystis*

(**a**) The structure consists of a central parallel β-sheet comprising seven β-strands, surrounded by nine α-helices. The 33-residue insertion (red) is unique to POR and is proposed to be involved in Pchlide (green) binding. (**b**) Three-dimensional model of the POR-catalysed reaction based on the structural homology model of POR [29] and the proposed mechanism of hydride and proton transfers. The proton at the C-18 position of Pchlide is derived from Tyr-189 (numbering in *Synechocystis* POR, cyan) and the hydride transferred to the C-17 position is derived from the pro-S face of NADPH (yellow). Modified from [23] with permission. © 2005 Elsevier.



[27,28]. Unfortunately, the structure of POR has not been resolved yet, but a homology model of the enzyme from *Synechocystis* was constructed using the tyrosine-dependent oxidoreductase family as a template [29] (Figure 1).

By illuminating POR–substrate complexes at low temperatures, intermediate states in the reaction pathway can be trapped and characterized by CW (continuous wave) fluorescence and absorption measurements. Consequently, by using this method, several spectroscopic intermediates before the formation of Chlide were detected, the first being a non-fluorescent intermediate with a broad absorbance band at 696 nm [30]. By using a combination of EPR, ENDOR (electron nuclear double resonance) and Stark spectroscopies, in conjunction with low-temperature absorbance spectroscopy, this intermediate was shown to be a charge-transfer complex resulting from hydride transfer from the NADPH molecule to the C-17 position of Pchlide [31]. Further intermediates were formed only above the glass transition temperature (200 K), suggesting a role for domain movements and/or reorganization of the protein, and were shown to correspond to a series of ordered product release and cofactor binding events [30–33]. Recently, the latter were time-resolved (at room temperature), and release of $NADP^+$ was observed to occur with rates of 1.2 and 0.2 ms^{-1} and was followed by the binding of NADPH and release of Chlide with a rate constant of 20 s^{-1} [34].

Ultrafast catalytic processes in POR

In addition, femtosecond transient absorption measurements have previously been used to study the initial ultrafast steps that are associated with formation of Chlide at room temperature. In these experiments [35], the Pchlide molecule was excited at 475 nm by a 30 fs laser pulse, and the resulting absorption changes were detected in the 600–750 nm spectral range. Immediately after excitation, a negative signal was observed due to the bleached absorption and stimulated emission of Pchlide, reaching a peak at approx. 640 nm. After a further few picoseconds, a negative signal, corresponding to stimulated emission, appeared at approx. 674 nm. A global analysis of all time traces collected at 250 different wavelengths revealed that the appearance of this emission occurred with two time constants of 3 and 400 ps, and was correlated to changes in the region of the Pchlide-stimulated emission and bleached absorption region. The absence of this negative signal in measurements on Pchlide in solution and in a mutant (Y189F) in which the putative proton donor, Tyr-189, was replaced by a phenylalanine residue, led to the conclusion that this was a catalytic product. The exact nature of this product state (I^*_{675}) remains to be determined, for example by the observation of kinetic isotope effects, a full exploration of subsequent events on the nano- to milli-second timescale or by investigations in the mid-IR spectral range, which in general lead to more structural information. Most likely, it represents an intermediate product, in which the Pchlide molecule forms strong hydrogen bonds with molecules in its environment, as was recently proposed to occur in Pchlide in solution using time-dependent density functional theory [36].

Ultrafast dynamics of Pchilde in solution

Transient absorption and time-resolved fluorescence measurements on the Pchlide molecule in several solvents revealed Pchlide to be an intrinsically reactive molecule [37–39]: the absorption difference spectra showed an initial blue shift, followed by a progressive red shift of the signal. Time-resolved emission spectra demonstrated that the main band at 640 nm follows different dynamics from the shoulder at 700 nm. Normally, this shoulder would be assigned to a vibrational progression, in which case it would show the same dynamics as the main band. The complex dynamics were interpreted with a model describing the excited-state processes in terms of a branching of the initially excited-state population into a reactive and a non-reactive path. The reactive path entailed the formation of a state with intramolecular charge-transfer character in ~25 ps and subsequent decay in 200 ps; the non-reactive path displayed vibrational relaxation in ~4 ps only. The reactive pathway was populated only in polar solvents. On the basis of time-dependent density functional theory, these results were interpreted in a slightly different way [36]: the S1 states of both the isolated Pchlide and its co-ordinated and hydrogen-bonded complexes were found to be of intramolecular charge transfer character. Since only the 4 ps process was common to all solvents, this time constant was assigned to ultrafast vibrational relaxation and vibrational energy redistribution of the initially excited S1 state with ICT (intramolecular charge transfer) character, followed for polar solvents by site-specific solvation, resulting in a strongly co-ordinated and hydrogen-bonded intermediate state, in 25 ps.

Figure 2 | Transient absorption traces recorded at the wavelength of Pchlide bleaching and stimulated emission (640 nm) and I^*_{675} emission region (677 nm), for subsequent scans

The traces at 677 nm show initially (black line) only the Pchlide excited-state absorption, which decays in a few nanoseconds; in later scans, I^*_{675} emission appears on a picosecond timescale (blue, green and magenta), and progressively more accumulated Chlide appears as a bleach of absorption at $t = 0$ (cyan and yellow).

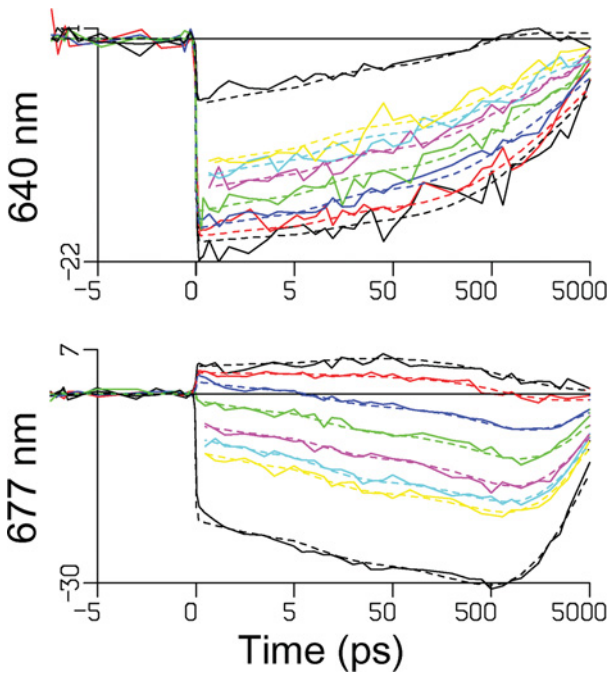
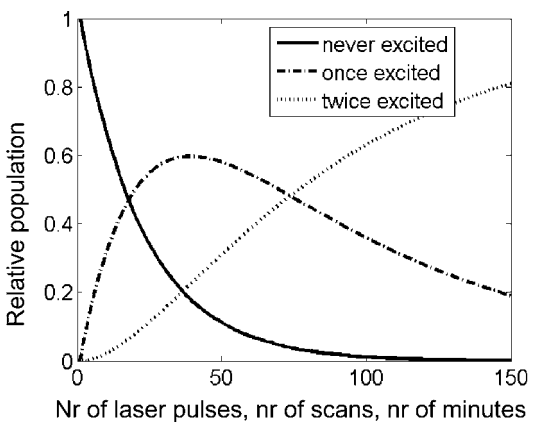
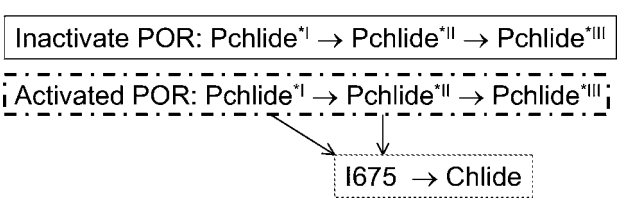


Figure 3 | Multiple excited complexes

Simulation of the dependence of the population of never-excited, once-excited and twice-excited POR–Pchlide complexes, on the number of applied laser pulses, for an excitation density of 0.045 per pulse. The laser-induced dynamics in complexes that had not been excited before corresponds to intrinsic photochemistry of Pchlide only. In complexes that had seen one prior excitation, subsequent excitation leads to the appearance of the intermediate I^*_{675} on a picosecond timescale (with rates of ~300 and 3.7 ns^{-1}). Since this intermediate converts into Chlide on a slower timescale, in complexes that had been excited twice before, excitation of Chlide and intrinsic Chlide photochemistry occurs.





Activation of the POR enzyme

In a subsequent study of the POR–Pchlide–NADPH complex, the ultrafast reaction dynamics were analysed in more detail to investigate the response of the enzyme to a single excitation pulse [40]. A Lissajous sample scanner was employed that moves the sample at a rate sufficient to provide each laser pulse out of the 1 kHz pulse train with a fresh spot on the sample and puts the same sample spot in the laser focus after approx. 1 min. A high detection-sensitivity of the laser pump–probe absorption difference setup (described in [41]) ensured that a full dataset, consisting of 48 time points and 256 spectral points, could be collected within 1 min. Therefore datasets could be collected on excitation by a first pulse, a second, a third etc. For full experimental details, see [40].

The dynamics of the POR–substrate complex proved to be very strongly dependent on the number of pulses applied to the sample (Figure 2). Following a single laser pulse, only minor dynamics in the Pchlide region were observed, with no I^*_{675} formation. However, after further laser pulses, stimulated emission from I^*_{675} appeared at 675 nm on the same timescale as reported previously [35]. To ensure a coherent description of all 55 datasets, collected as a function of the number of excitation pulses applied to the sample, the data had to be fitted to a model that included not only the dynamics and the 'delayed' appearance of I^*_{675}, but also the decreasing amount of Pchlide and the increasing amount of Chlide in the sample during the experiments. A good fit could be obtained with a model in which the POR enzymes were divided into two populations: one population that is inactive and shows intrinsic Pchlide photochemistry [38], but does not lead to product, and an active population that also shows the formation of I^*_{675} (Figure 3). The relative concentrations of inactive Pchlide, active Pchlide and Chlide that were obtained from this analysis followed profiles consistent with populations of non-, single- or twice-excited pigments. This suggested that one photon is needed to activate the POR complex and a second photon then induces catalysis. To see whether structural changes in the POR–NADPH–PChlide complex were associated with this activation, rapid-scan FTIR (Fourier-transform infrared) experiments were performed [40]. The initial laser shots induced absorption changes associated mainly with protein bands, amide I and II; subsequent laser shots induced absorption changes that could be assigned to the disappearance of NADPH and PChlide and the appearance of $NADP^+$ and Chlide. This showed (i) that indeed the first photon induces a conformational change in the POR enzyme that switches it from inactive to active, and (ii) that,

when in the active state, a second photon can induce catalysis, which leads to Chlide formation with a quantum yield of 0.3.

Conclusions

The combination of a convenient model system, such as the light-dependent POR enzyme, and a carefully designed kinetic experiments allows the real-time observation of conformational changes leading to activation and the subsequent catalytic reactions. The case of POR clearly demonstrates the importance of conformational changes for catalysis in enzymes. We expect that further experiments on POR will lead to a full identification of the reaction path at room temperature and the identification of the early intermediate state(s) involved, and a better understanding of which structural changes lie at the origin of the activation process. The resolution of the structure of POR, either by X-ray diffraction or by NMR techniques, will undoubtedly be important in this process.

Funding

This research was supported by the Netherlands Organisation for Scientific Research through the Dutch Foundation for Earth and Life Sciences [investment grant number 834.01.002]. M.L.G. is grateful to the Netherlands Organisation for Scientific Research through the Dutch Foundation for Earth and Life Sciences for providing financial support with a long-term fellowship [grant number 831.00.004]. D.J.H. and C.N.H. gratefully acknowledge support from the Biotechnology and Biological Sciences Research Council [grant number BB/D015413/1].

References

1 Benkovic, S.J. and Hammes-Schiffer, S. (2003) A perspective on enzyme catalysis. Science **301**, 1196–1202
2 Villa, J. and Warshel, A. (2001) Energetics and dynamics of enzymatic reactions. J. Phys. Chem. B **105**, 7887–7907
3 Masgrau, L., Roujeinikova, A., Johannissen, L.O., Hothi, P., Basran, J., Ranaghan, K.E., Mulholland, A.J., Sutcliffe, M.J., Scrutton, N.S. and Leys, D. (2006) Atomic description of an enzyme reaction dominated by proton tunneling. Science **312**, 237–241
4 Kohen, A., Cannio, R., Bartolucci, S. and Klinman, J.P. (1999) Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase. Nature **399**, 496–499
5 Eisenmesser, E.Z., Millet, O., Labeikovsky, W., Korzhnev, D.M., Wolf-Watz, M., Bosco, D.A., Skalicky, J.J., Kay, L.E. and Kern, D. (2005) Intrinsic dynamics of an enzyme underlies catalysis. Nature **438**, 117–121
6 Agarwal, P.K. (2005) Role of protein dynamics in reaction rate enhancement by enzymes. J. Am. Chem. Soc. **127**, 15248–15256
7 Wang, L., Goodey, N.M., Benkovic, S.J. and Kohen, A. (2006) Coordinated effects of distal mutations on environmentally coupled tunneling in dihydrofolate reductase. Proc. Natl. Acad. Sci. U.S.A. **103**, 15753–15758
8 Karplus, M. (2000) Aspects of protein reaction dynamics: Deviations from simple behavior. J. Phys. Chem. B **104**, 11–27
9 Cui, Q. and Karplus, M. (2008) Allostery and cooperativity revisited. Protein Sci. **17**, 1295–1307
10 Doster, W. (2008) The dynamical transition of proteins, concepts and misconceptions. Eur. Biophys. J. Biophys. Lett. **37**, 591–602
11 Samuni, U., Dantsker, D., Roche, C.J. and Friedman, J.M. (2007) Ligand recombination and a hierarchy of solvent slaved dynamics: the origin of kinetic phases in hemeproteins. Gene **398**, 234–248
12 Frauenfelder, H., McMahon, B.H., Austin, R.H., Chu, K. and Groves, J.T. (2001) The role of structure, energy landscape, dynamics, and allostery in the enzymatic function of myoglobin. Proc. Natl. Acad. Sci. U.S.A. **98**, 2370–2374
13 Lu, H.P., Xun, L.Y. and Xie, X.S. (1998) Single-molecule enzymatic dynamics. Science **282**, 1877–1882
14 Eisenmesser, E.Z., Bosco, D.A., Akke, M. and Kern, D. (2002) Enzyme dynamics during catalysis. Science **295**, 1520–1523
15 Flomenbom, O., Velonia, K., Loos, D., Masuo, S., Cotlet, M., Engelborghs, Y., Hofkens, J., Rowan, A.E., Nolte, R.J.M., Van der Auweraer, M. et al. (2005) Stretched exponential decay and correlations in the catalytic activity of fluctuating single lipase molecules. Proc. Natl. Acad. Sci. U.S.A. **102**, 2368–2372
16 Okamura, M.Y., Paddock, M.L., Graige, M.S. and Feher, G. (2000) Proton and electron transfer in bacterial reaction centers. Biochim. Biophys. Acta **1458**, 148–163
17 Paddock, M.L., Feher, G. and Okamura, M.Y. (2003) Proton transfer pathways and mechanism in bacterial reaction centers. FEBS Lett. **555**, 45–50
18 Wraight, C.A. (2004) Proton and electron transfer in the acceptor quinone complex of photosynthetic reaction centers from *Rhodobacter sphaeroides*. Front. Biosci. **9**, 309–337
19 Byrdin, M., Sartor, V., Eker, A.P.M., Vos, M.H., Aubert, C., Brettel, K. and Mathis, P. (2004) Intraprotein electron transfer and proton dynamics during photoactivation of DNA photolyase from *E. coli*: review and new insights from an 'inverse' deuterium isotope effect. Biochim. Biophys. Acta **1655**, 64–70
20 Aubert, C., Vos, M.H., Mathis, P., Eker, A.P.M. and Brettel, K. (2000) Intraprotein radical transfer during photoactivation of DNA photolyase. Nature **405**, 586–590
21 Belyaeva, O.B. and Litvin, F.F. (2007) Photoactive pigment–enzyme complexes of chlorophyll precursor in plant leaves. Biochemistry **72**, 1458–1477
22 Masuda, T. and Takamiya, K. (2004) Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. Photosynth. Res. **81**, 1–29
23 Heyes, D.J. and Hunter, C.N. (2005) Making light work of enzyme catalysis: protochlorophyllide oxidoreductase. Trends Biochem. Sci. **30**, 642–649
24 Lebedev, N. and Timko, M.P. (1998) Protochlorophyllide photoreduction. Photosynth. Res. **58**, 5–23
25 Griffiths, W.T. (1978) Reconstitution of chlorophyllide formation by isolated etioplast membranes. Biochem. J. **174**, 681–692
26 Wilks, H.M. and Timko, M.P. (1995) A light-dependent complementation system for analysis of NADPH–protochlorophyllide oxidoreductase: identification and mutagenesis of 2 conserved residues that are essential for enzyme-activity. Proc. Natl. Acad. Sci. U.S.A. **92**, 724–728
27 Valera, V., Fung, M., Wessler, A.N. and Richards, W.R. (1987) Synthesis of 4R-tritium and 4S-tritium labeled NADPH for the determination of the coenzyme stereospecificity of NADPH–protochlorophyllide oxidoreductase. Biochem. Biophys. Res. Commun. **148**, 515–520
28 Begley, T.P. and Young, H. (1989) Protochlorophyllide reductase. 1. Determination of the regiochemistry and the stereochemistry of the reduction of protochlorophyllide to chlorophyllide. J. Am. Chem. Soc. **111**, 3095–3096
29 Townley, H.E., Sessions, R.B., Clarke, A.R., Dafforn, T.R. and Griffiths, W.T. (2001) Protochlorophyllide oxidoreductase: a homology model examined by site-directed mutagenesis. Proteins Struct. Funct. Genet. **44**, 329–335
30 Heyes, D.J., Ruban, A.V., Wilks, H.M. and Hunter, C.N. (2002) Enzymology below 200 K: the kinetics and thermodynamics of the photochemistry catalyzed by protochlorophyllide oxidoreductase. Proc. Natl. Acad. Sci. U.S.A. **99**, 11145–11150
31 Heyes, D.J., Heathcote, P., Rigby, S.E.J., Palacios, M.A., van Grondelle, R. and Hunter, C.N. (2006) The first catalytic step of the light-driven enzyme protochlorophyllide oxidoreductase proceeds via a charge transfer complex. J. Biol. Chem. **281**, 26847–26853
32 Heyes, D.J. and Hunter, C.N. (2004) Identification and characterization of the product release steps within the catalytic cycle of protochlorophyllide oxidoreductase. Biochemistry **43**, 8265–8271
33 Heyes, D.J., Ruban, A.V. and Hunter, C.N. (2003) Protochlorophyllide oxidoreductase: 'dark' reactions of a light-driven enzyme. Biochemistry **42**, 523–528
34 Heyes, D.J., Sakuma, M. and Scrutton, N.S. (2007) Laser excitation studies of the product release steps in the catalytic cycle of the light-driven enzyme, protochlorophyllide oxidoreductase. J. Biol. Chem. **282**, 32015–32020

35 Heyes, D.J., Hunter, C.N., van Stokkum, I.H.M., van Grondelle, R. and Groot, M.L. (2003) Ultrafast enzymatic reaction dynamics in protochlorophyllide oxidoreductase. Nat. Struct. Biol. **10**, 491–492
36 Zhao, G.J. and Han, K.L. (2008) Site-specific solvation of the photoexcited protochlorophyllide a in methanol: formation of the hydrogen-bonded intermediate state induced by hydrogen-bond strengthening. Biophys. J. **94**, 38–46
37 Schmitt, M., Dietzek, B., Hermann, G. and Popp, J. (2007) Femtosecond time-resolved spectroscopy on biological photoreceptor chromophores. Laser Photonics Rev. **1**, 57–78
38 Dietzek, B., Kiefer, W., Hermann, G., Popp, J. and Schmitt, A. (2006) Solvent effects on the excited-state processes of protochlorophyllide: a femtosecond time-resolved absorption study. J. Phys. Chem. B **110**, 4399–4406
39 Dietzek, B., Maksimenka, R., Siebert, T., Birckner, E., Kiefer, W., Popp, J., Hermann, G. and Schmitt, M. (2004) Excited-state processes in protochlorophyllide a: a femtosecond time-resolved absorption study. Chem. Phys. Lett. **397**, 110–115
40 Sytina, O.A., Heyes, D.J., Hunter, C.N., Alexandre, M.T.A., van Stokkum, I.H.M., van Grondelle, R. and Groot, M.L. (2008) Conformational changes in an ultrafast light-driven enzyme determine catalytic. Nature **456**, 1001–1004
41 Groot, M.L., van Wilderen, L. and Di Donato, M. (2007) Time-resolved methods in biophysics. 5. Femtosecond time-resolved and dispersed infrared spectroscopy on proteins. Photochem. Photobiol. Sci. **6**, 501–507

Received 8 October 2008
doi:10.1042/BST0370387