A simple procedure for the photoregulation of chymotrypsin activity

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A convenient and rapid method for the photo-regulation of the proteolytic enzyme α-chymotrypsin is described. When α-chymotrypsin is coated with photolytic 1-(2-nitrophenyl)ethanol residues this not only markedly reduces the capability of the enzyme to digest both of the small substrates *N*-benzoyl-L-tyrosine ethyl ester and *N*-succinyl-L-phenylalanine *p*-nitroanilide, but also completely inhibits the enzyme's proteolytic activity. The inactivated α-chymotrypsin can then be reactivated under physiological conditions, when and where it is required, by exposure to UV-A light. These results further demonstrate that 1-(2-nitrophenyl)ethanol coated proteins can often be used as light sensitive biological switches as a simple alternative to site directed procedures.

Introduction

Many important cellular and biological studies have depended on the synthesis and use of 'caged' compounds. On irradiation with light the photolabile protecting groups are removed and biological activity is regenerated both when and where it is required.[1,2] Unfortunately, however, relatively few studies have tackled the more complex goal of 'caging' proteins.[3,4] It is extremely difficult to photo-regulate the activity of enzymes unless the enzyme is blocked in a site specific manner, and this often requires more complex techniques such as unnatural amino acid mutagenesis.[5–8] Indeed it is often much easier to block an enzyme modulator/catalyst than the enzyme itself.[9,10] A non site-specific 'caging' procedure which could be used to photo-regulate the activity of enzymes would be a very useful biological tool.

The serine proteases, thrombin, factor Xa and chymotrypsin have been caged by the addition of cinnamate derivatives. These were tailored to fit the enzyme's active site and were subsequently found to inhibit the enzyme by acylating the site's essential serine residue.[11–13] Some of the inactive acylated enzymes were not very stable and hydrolysed back to the active enzyme fairly rapidly.[13] However others could be considered to be stable indefinitely. On UV-irradiation at 366 nm for 5–10 min up to 80% of the enzyme's activity was recoverable.

We here discuss an alternative and simpler procedure for the reversible inhibition of α-chymotrypsin. This is based on a generically applicable method[14] we have previously shown to be capable of reversibly inactivating antibodies.[15,16] α-Chymotrypsin is inactivated by simply coating it with photocleavable 1(2-nitrophenyl)ethanol (NPE) residues *via* photolabile carbamate linkages[17] (Fig. 1). On irradiation with UV light at 365 nm the 2-nitrobenzyl residues transform into unstable 2-nitroso derivatives[3] which then break down into nitrosoacetophenone and CO_2 releasing the enzyme in its natural form. This restoration of enzyme activity, when taken in conjunction with the regulation of antibody activity[15,16] further demonstrates that this NPE coating procedure is generally applicable to protein photo-regulation. Such conjugates could be used as light controlled biological switches in numerous fundamental and therapeutic applications.

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Experimental

α-Chymotrypsin (Type II) was obtained from Sigma, Poole, UK, the substrates SPNA and BTEE from ICN Biochemicals, Thame, UK, and all other chemicals from Fluka, Poole, UK.

Coupling of 1(2-nitrophenyl)ethanol groups

1(2-Nitrophenyl)ethanol was coupled covalently to α-chymotrypsin (Fig. 1) as previously described for BSA and IgG.[14–16] Ten μl of 1-(2-nitrophenyl)ethanol (NPE) was reacted at room temperature with 7.8 μl of di-phosgene in 250 μl of dry dioxan in the presence of 5.2 μl pyridine as a catalyst. The mixture was left for 15 min before unreacted materials were evaporated away in a stream of nitrogen. The highly reactive 1-(2-nitrophenyl)-ethoxycarbonylchloride was then resuspended in 250 μl dioxan and 0, 5, 10, 20 and 50 μl aliquots of this solution were added to 1 ml portions of a 1.4 mg ml^{-1} solution of α-chymotrypsin in 0.1 M $NaHCO_3$ pH 8.3. The mixtures were left overnight for coupling to go to completion then dialysed against 0.9% NaCl at 4 °C for 16 h. White insoluble reaction products were removed by micro-centrifugation for 15 min at 13 000 *g* and the NPE-coated chymotrypsin conjugates were left in the clear supernatant.

The protein concentration[18] and the absorbance (OD280 nm) value of each sample were measured. As the absorbance of uncoated α-chymotrypsin was known from the control sample, and a solution of 50 μg ml^{-1} NPE had an OD280 nm value of 1.0, the amount of NPE, and hence the average number of NPE residues bound to each α-chymotrypsin molecule could be estimated from the relative increase in the absorbance value of each sample. It is assumed that the caging of the enzyme with NPE does not affect the protein assay. The fact that the OD values decrease (through loss of NPE), whilst the protein concentration remains constant, following irradiation and dialysis of the caged samples, lends credence to this assumption.

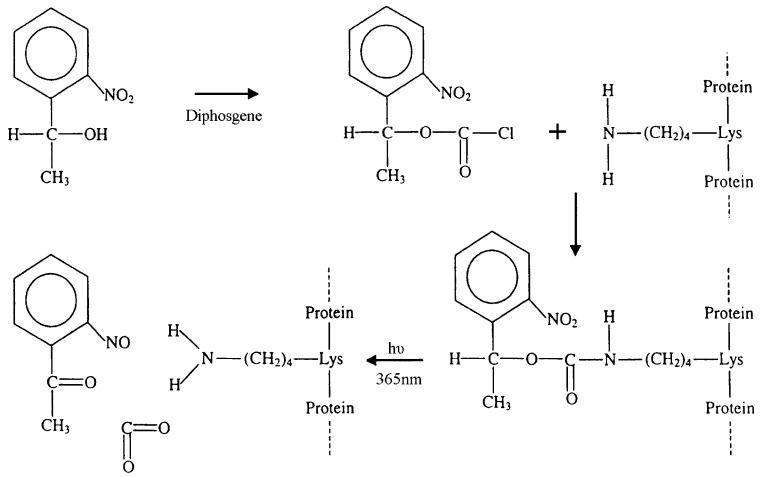

Fig. 1 The proposed reaction mechanism for the coupling and decoupling of 1(2-nitrophenyl)ethanol to proteins.

Enzymic activity

Two small substrates were initially used in the determination of α-chymotrypsin activity, *N*-benzoyl-L-tyrosine ethyl ester (BTEE)[19] and *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA).[20] In both assays sample activity (both uncoated and NPE-coated samples), was measured both before and after irradiation with UV light.

The BTEE assay was carried out in a quartz cuvette. 500 μl of 1.07 mM BTEE in 50% methanol was added to 300 μl of 80 mM Tris-HCl buffer pH 7.8 containing 100 mM $CaCl_2$. 200 μl of a solution of each α-chymotrypsin sample (0.1 mg ml^{-1} in 0.9% NaCl) was then added to the cuvette and the rate of reaction was measured at 256 nm for 90 s. The SPNA assay was carried out in a 96 well ELISA plate. SPNA was first dissolved in 50% methanol then an equal volume of 200 mM triethanolamine buffer (TRA) pH 7.8 containing 100 mM $CaCl_2$ was added. 200 μl of this solution was then pipetted into each well of an ELISA plate. 33 μl of a 0.1 mg ml^{-1} solution of each α-chymotrypsin sample was then added to each well and the absorbance of the samples was measured at 405 nm at 0, 15 and 30 min after their addition.

Proteolytic activity

Bovine serum albumin (BSA) was added to both NPE-coated and uncoated α-chymotrypsin samples to determine how the NPE coating affected the ability of the enzyme to digest large protein molecules.

250 μl of uncoated and NPE-coated α-chymotrypsin samples (at 0.5 mg ml^{-1}) were irradiated with UV-A light and 50 μl aliquots were removed after 0, 2, 5 or 10 min. Each aliquot had 100 μl of BSA (0.5 mg ml^{-1} in Tris buffer pH 7.8 as above) added to it and the samples were then left for 15 min for digestion to proceed. 100 μl of denaturing electrophoresis sample buffer[21] containing 10% SDS was then added and the samples were immediately boiled for 5 min to stop the reaction. Equal quantities of each sample (30 μl) were then separated by discontinuous electrophoresis in 9% polyacrylamide gels.[21] After staining with Coomassie blue dye the amount of undigested BSA remaining in each sample was determined by scanning laser densitometry.

Photolysis of conjugates

α-Chymotrypsin samples were diluted to 0.1 mg ml^{-1} in 0.9% NaCl in quartz cuvettes. They were then irradiated with UV light for a range of times at room temperature. After irradiation the samples were then dialysed for 16 h against 0.9% NaCl to remove photocleaved products and the protein concentrations and OD280 nm values were remeasured. The reduction in OD values to those of uncoated chymotrypsin not only demonstrated that the NPE was being removed, but also proved that its photolysed reaction products were not binding to the chymotrypsin. A Spectroline EN-16/F UV lamp (Spectronics Corporation, New York) with an emission peak of 365 nm was used as the source of the UV light. The total UV-A irradiance of the lamp was 5.45 mW cm^{-2} at the working distance of 0.3 cm.[22] The α-chymotrypsin samples that were used to digest BSA were irradiated with a VL-206BL UV-A lamp (2 × 6 W tubes) which had a total UV-A irradiance of approximately 16 mW cm^{-2} at a distance of 1 cm.

Results

An average of 0, 1, 4, 7 and 9 NPE residues bound to each α-chymotrypsin molecule on the addition of 0, 5, 10, 20 and 50 μl NPE-carbonyl chloride to each α-chymotrypsin sample. The enzymic activity of each sample was then measured in three separate assays with each substrate and the results of a typical assay with each substrate are given in Tables 1 and 2. In all cases enzymic activity is expressed as a percentage of that obtained with uncoated unirradiated native enzyme.

When BTEE was used as the substrate (Table 1) the activity of the enzyme was found to decrease from 100% to as little as 7% of the native value as the number of coupled NPE groups increased. However when the NPE groups were removed by exposure to UV-light the majority of the enzyme's activity could be restored. The activity of the α-chymotrypsin sample coated with 1 NPE residue increased from 65 to 96% on 15 min UV-irradiation, whilst the sample coated with 9 NPE residues increased from 7 to 78% (a 10 fold increase).

When SPNA was used as the substrate (Table 2) similar results were obtained. The enzyme activity progressively decreased as the number of NPE groups coating the enzyme increased and

Table 1 The photoregulation of α-chymotrypsin activity (using BTEE)[a]

NPE added/μl	No. NPE residues	UV-A exposure/min			
		0	5	10	15
0	0	100	106	106	118
5	1	65	79	81	96
10	4	60	83	89	116
20	7	26	72	88	98
50	9	7	52	74	78

[a] The figures for α-chymotrypsin activity from each NPE-coated sample represent the increase in OD 256 nm value (due to the release of *N*-benzoyl-L-tyrosine) given by each sample expressed as a percentage of the increased OD 256 nm value given by control uncoupled unirradiated α-chymotrypsin.

Table 2 The photoregulation of α-chymotrypsin activity (using SPNA)[a]

NPE added/μl	No. NPE residues	UV-A exposure/min		
		0	15	30
0	0	100	100	100
5	1	66	79	82
10	4	43	78	82
20	7	25	61	74
50	9	15	80	91

[a] The figures for α-chymotrypsin activity from each NPE-coated sample represent the increase in OD 405 nm value (due to *p*-nitroaniline release) given by each sample expressed as a percentage of the increased OD 405 nm value given by control uncoupled unirradiated α-chymotrypsin.

Table 3 The removal of NPE residues from α-chymotrypsin by UV-A light

NPE added/μl	No. of NPE residues after UV exposure			
	0 min	2 min	5 min	10 min
0	0	0	0	0
20	5.4	1.1	0.3	0
50	10.5	2.9	1.5	0.7

considerable enzymic activity could be restored on irradiation with UV light for 15 min. This could be increased still further (especially in the samples coated with 7 or 9 residues) on 30 min irradiation.

As the above initial studies had shown that the NPE-coating could reversibly inhibit the ability of α-chymotrypsin to digest both small substrates, the ability of NPE-coated α-chymotrypsin to digest a larger protein substrate (BSA), a more biologically relevant assay, was then examined. A new UV-A lamp was also used in an attempt to reduce irradiation times.

α-Chymotrypsin was again coated with 20 or 50 μl NPE. Table 3 shows that after 0, 2, 5 and 10 min irradiation an average of 5.4, 1.1, 0.3 and 0 residues of NPE and 10.5, 2.9, 1.5 and 0.7 residues of NPE remained on each α-chymotrypsin molecule in each sample. The vast majority of the NPE was therefore released by 2 min irradiation and very few residues were present after 5 min UV-A treatment. The amount of BSA digested by each sample is shown in Fig. 2 and quantified in Table 4. The α-chymotrypsin sample coated with 5.4 residues was initially inhibited by around 50% but was restored to full activity by 2 min irradiation. The highly coated sample appeared to show total inhibition followed by the rapid and total recovery of α-chymotrypsin activity. Due to the importance of the latter result, another sample of highly coated α-chymotrypsin was prepared (50 μl NPE, 11 residues of NPE per α-chymotrypsin molecule). This was irradiated along with another aliquot of the previous highly coated sample. The results are shown in Fig. 3 and Table 5. Complete inhibition followed by rapid and total recovery of α-chymotrypsin activity was found in each sample.

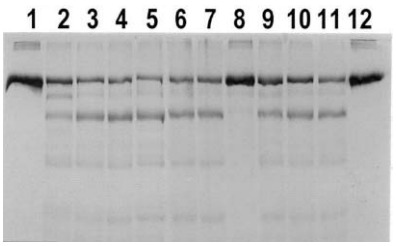

Fig. 2 A comparison of the proteolytic activity of α-chymotrypsin and NPE coated α-chymotrypsin samples before and after irradiation with UV-A light. All lanes were loaded with 30 μl of sample containing identical amounts of BSA (6 μg). Lanes 1 and 12 were control lanes containing undigested BSA alone. Lanes 6 and 7 show the amount of BSA digested by uncoated chymotrypsin after 0 and 10 min irradiation. Lanes 2–5 show the amount of BSA digested by NPE(5.4 residues)-chymotrypsin after 0, 2, 5 and 10 min irradiation. Lanes 8–11 show the amount of BSA digested by NPE(10.5 residues)-chymotrypsin after 0, 2, 5 and 10 min irradiation.

Discussion

The above data demonstrate that chymotrypsin can be coated with up to 11 residues of NPE which completely deactivates the enzyme. This NPE coat can be rapidly removed, and the enzyme reactivated, by illumination with light from a hand held UV-A lamp. Small differences in coating are sometimes

Table 4 The photoregulation of α-chymotrypsin proteolytic activity[a]

NPE added/μl	No. NPE residues	UV-A exposure/min							
		0		2		5		10	
0	0	100	100	103	nd	97	nd	95	89
20	5.4	48	69	97	99	106	97	105	105
50	10.5	0	0	67	63	80	87	100	103

[a] The figures for α-chymotrypsin activity from each NPE-coated sample represent the amount of BSA digested by each sample expressed as a percentage of the amount of BSA digested by control uncoupled unirradiated α-chymotrypsin. Values are given for densitometry of the same samples on 2 different gels.

Table 5 The photoregulation of two highly coated NPE-chymotrypsin samples[a]

NPE added/μl	No. NPE residues	UV-A exposure/min			
		0	2	5	10
0	0	100	104	nd	96
50	11.0	0	73	100	96
50	10.5	0	65	99	96

[a] The figures for α-chymotrypsin activity from each NPE-coated sample represent the amount of BSA digested by each sample expressed as a percentage of the amount of BSA digested by control uncoupled unirradiated α-chymotrypsin.

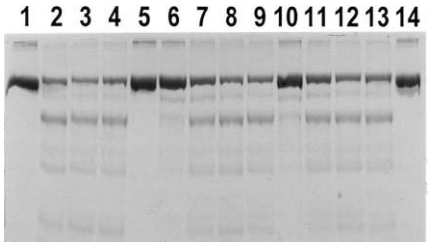

Fig. 3 The digestion of BSA by two heavily coated NPE-chymotrypsin samples before and after irradiation with UV-A light. All lanes were again loaded with 30 μl of sample containing identical amounts of BSA (6 μg). Lanes 1, 5 and 14 were control lanes containing undigested BSA alone. Lanes 2–4 show the amount of BSA digested by uncoated chymotrypsin after 0, 5 and 10 min irradiation. Lanes 6–9 and lanes 10–13 show the amount of BSA digested by two heavily coated NPE-chymotrypsin samples (10.5 and 11 residues) after 0, 2, 5 and 10 min irradiation respectively.

obtained, *e.g.* 5.4/7 residues by the addition of 20 μl of the 1-(2-nitrophenyl)ethoxycarbonylchloride and 9/10.5/11.0 residues using 50 μl. This effect probably occurs because the highly reactive white crystals of 1-(2-nitrophenyl)ethoxycarbonylchloride are only suspended, and not completely solubilised, in dry dioxan. It is therefore difficult to guarantee the addition of exactly the same amount of reactant in each experiment. Room temperature fluctuations during each overnight coupling may also alter the final extent of coupling.

Whilst coating α-chymotrypsin with NPE groups effectively inhibits the enzyme in such a way as to provide a photo-regulatable enzyme, the mechanism of inhibition is not yet clear. It could arise through the NPE groups binding in or close by the active site of the enzyme and/or coupling to surface lysine residues in the protein backbone and thus changing the overall hydrophobicity and/or conformation of the enzyme. It appears unlikely that this effect is the result of NPE coupling to the surface lysine residues as the blocking of all 13 surface lysine groups with *o*-methylisourea had no detectable effect on α-chymotrypsin activity.[23] An alternative and more probable explanation of the results presented here would be that the NPE groups are binding to (and blocking) the terminal ile16 residue of the α-chymotrypsin B chain. This residue is known to be critical to α-chymotrypsin activity and is formed when inactive chymotrypsinogen is converted to active enzyme by the specific hydrolysis of the arg15–ile16 bond.[24] If NPE does bind with some specificity to ile16 then this would easily explain why 35% of the enzyme activity is inhibited when an average of only 1 NPE group is coupled to each enzyme molecule.

The precise nature of this effect is to some extent unimportant, as long as activity is regained on removal of the caging groups. This work demonstrates that α-chymotrypsin as well as antibody preparations[15,16,25] can be rendered inactive with a 2-nitrobenzyl coat then reactivated at will by irradiation with UV light. This simple coating procedure operates efficiently in aqueous solutions, does not require site specific targeting or the synthesis of enzyme specific reagents,[5–8,11–13] and photo-recovery of activity is very rapid in therapeutic terms. Perhaps more importantly, the low power of the lamps means that reactivations can be carried out in the presence of cells and tissues without damaging them. We did not examine how short photolysis times could be with a more powerful lamp. Kossel *et al.*[25] reduced their reactivation times from >3 h with a hand held lamp, to seconds, utilising pulses from a focused 100 W mercury arc lamp. This allowed them to study synaptic potentiation in brain slices.

The relatively simple nature of this procedure makes it an attractive starting point prior to attempting more complex site specific molecular biological approaches. We are convinced that photo-switching procedures such as this will play an essential part in both diagnostic and clinical medicine as well as providing a useful tool for a better understanding of basic cellular reactions. We are currently using this photo-switching procedure to develop methods to deliver therapeutic proteins more accurately.

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References

1 G. Marriott, Caged Compounds, *Methods Enzymol.*, 1998, **291**, 1–515.
2 *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*, ed. M. Goeldner and R. Givens, Wiley-VCG, Weinheim, 2005.
3 K. Curley and D. S. Lawrence, Light activated proteins, *Curr. Opin. Chem. Biol.*, 1999, **3**, 84–88.
4 S. Loudwig and H. Bayley, Light activated proteins: An overview, in *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*, ed. M. Goeldner and R. Givens, Wiley-VCH, Weinheim, 2005, pp. 253–304.
5 E. J. Petersson, G. S. Brandt, N. M. Zacharias, D. A. Dougherty and H. A. Lester, Caging of proteins through unnatural amino-acic mutagenesis, *Methods Enzymol.*, 2003, **360**, 258–273.
6 K. Zou, S. Cheley, R. S. Givens and H. Bayley, Catalytic subunit of protein kinase A caged at the activating photothreonine, *J. Am. Chem. Soc.*, 2002, **124**, 6220–6229.
7 T. Hiraoka and I. Hamachi, Caged RNase: Photoactivation of the enzyme from perfect off state by site-specific incorporation of 2-nitrobenzyl moiety, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 13–15.
8 M. Endo, K. Nakayama and T. Majima, Design and synthesis of photochemically controllable restriction endoneuclease BamH1 by manipulating the salt-bridge network in the dimmer interface, *J. Org. Chem.*, 2004, **69**, 4292–4298.
9 L. Bedouet, H. Adenier, S. Pulvin, C. Bedel-Cloutour and D. Thomas, Recovery of the oxidative activity of caged bovine haemoglobin after UV photolysis, *Biochem. Biophys. Res. Commun.*, 2004, **320**, 939–944.
10 L. Peng and M. Goeldner, Photochemical enzyme regulation using caged enzyme modulators, in *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*, ed. M. Goeldner and R. Givens, Wiley-VCH, Weinheim, 2005, pp. 304–325.
11 N. A. Porter and J. D. Bruhnke, Photocoagulation of human plasma: Acyl serine proteinase photochemistry, *Photochem. Photobiol.*, 1990, **51**, 37–43.

12 N. A. Porter, J. W. Thuring and H. Li, Selective inhibition, separation, and purification of serine proteases: A strategy based on a photoremovable inhibitor, *J. Am. Chem. Soc.*, 1999, **121**, 7716–7717.
13 P. B. Jones and N. A. Porter, 2-Aroylbenzoyl serine proteases: Photoreversible inhibition or photoaffinity labelling, *J. Am. Chem. Soc.*, 1999, **121**, 2753–2761.
14 S. Thompson, J. A. Spoors, M.-C. Fawcett and C. H. Self, Photocleavable nitrobenyl-protein conjugates, *Biochem. Biophys. Res. Commun.*, 1994, **201**, 1213–1219.
15 S. Thompson, M.-C. Fawcett, J. A. Spoors and C. H. Self, The modulation of Protein A-IgG(Fc) binding by the reversible addition of 2-nitrobenzyl groups, *Biochem. Soc. Trans.*, 1995, **23**, 155S.
16 C. H. Self and S. Thompson, Light activatable antibodies: Models for remotely activatable proteins, *Nat. Med. (N. Y.)*, 1996, **2**, 817–820.
17 G. Marriott, Caged protein conjugates and light-directed generation of protein activity: Preparation, photoactivation, and spectroscopic characterization of caged G-actin conjugates, *Biochemistry*, 1994, **33**, 9092–9097.
18 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olsen and D. C. Klenk, Measurement of protein using bicinchoninic acid, *Anal. Biochem.*, 1985, **150**, 76–85.
19 B. C. W. Hummel, A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin, *Can. J. Biochem. Physiol.*, 1959, **37**, 1393–1399.
20 W. Nagel, F. Willig, W. Peschke and F. H. Schmidt, Uber die bestimmung von trypsin und chymotrypsin mit aminosaure-*p*-nitroanniliden, *Hoppe-Seylers Z. Physiol. Chem.*, 1965, **340**, 1–10.
21 S. Thompson and A. H. Maddy, Gel electrophoresis of erythrocyte membrane proteins,in *Red Cell Membranes-A Methodological Approach*, ed. J. D. Young and J. C. Ellory, Academic Press, New York, 1982, pp. 67–93.
22 C. H. Self, M.-C. Fawcett, J. A. Spoors, L. B. Pulman and S. Thompson, Studies on photocleavable nitrobenzyl-bovine serum albumin conjugates, *Biochem. Soc. Trans.*, 1995, **23**, 156S.
23 C. H. Chervenka and P. E. Wilcox, Chemical derivatives of chymotrypsinogen. II. Reaction with *o*-methylisourea, *J. Biol. Chem.*, 1956, **222**, 635–647.
24 P. B. Sigler, D. M. Blow, B. W. Matthews and R. Henderson, Structure of crystalline α-chymotrpsin, *J. Mol. Biol.*, 1968, **35**, 143–164.
25 A. H. Kossel, S. B. Cambridge, U. Wagner and T. Bonhoeffer, A caged Ab reveals an immediate/instructive effect of BDNF during hippocampal synaptic potentiation, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 14702–14707.

