phenol red or riboflavin and supplemented with 25 mM HEPES and 0.2% BSA. Solutions were maintained at 4°C until immediately before each experiment, when they were warmed to 37°C and supplemented with 50 mM PDBU and either 0.5 mM EGTA (50 μM free Ca2+) or 1 mM CaCl2 (1.5 mM total Ca2+).

**Transfection of the reporter genes.** NF-AT, NF-κB, Oct-1/OAP and IL-2 reporter constructs were provided by J. Goldberg and G. Crabtree and were derived from constructs described previously. Multimeric copies of NFAT (3 copies), NF-κB (4) or Oct-1/OAP (4) binding sites, linked to a minimal (non-inducible) IL-2 promoter (−74 to +47), were inserted between the Smad and HnIII sites in the multiple cloning region of the pGL-3 luciferase reporter vector (Promega). The construct with the intact IL-2 promoter region was made by inserting 371 bp of the IL-2 initiation region (−324 to +47) into the pGL-3 vector. The IL-8 reporter construct was provided by K. P. LeClair.

Transfection of 106 cells was by electroporation with 10 μg of the reporter vector, 1 μg of a vector containing large-T antigen and 2 μg of a vector encoding the transmembrane and extracellular domains of CD8. Large-T antigen was used to increase the number of copies of the reporter construct in each cell, and the CD8 construct was used to determine the transfection efficiency. Transfection efficiencies were 30–40%; viability following centrifugation to remove cells killed during electroporation was 85–95%. Experiments were conducted 24–48 h after transfection when the expression of reporter genes was maximal.

**Ca2+ clamp and Ca2+ measurements.** Transfected cells were loaded with 2 μM Fura-PE3 (Teflabs) for 1 h at 37°C in loading medium (RPMI 1640, 25 mM HEPES, 2% fetal bovine serum), washed, and incubated for another hour to allow complete de-esterification of the dye. Loaded cells (2–3 × 105) were allowed to adhere to a polylysine-coated laminar flow chamber and were placed on the heated stage of a Zeiss Axiovert 35 inverted microscope. The laminar flow chamber (60 μl volume) was connected to two heated reservoirs and impermeably activate CRAC channels. A computer-controlled solenoid valve (General Valve) was used to switch rapidly between the Ca2+-containing and Ca2+-free solutions flowing into the chamber and over the cells. The solution in the chamber was fully exchanged about once per second and was maintained at 37°C. Cells were stimulated for 3 h while [Ca2+] was measured every 5–10 s by video microscopy as described. Fura-PE3 was calibrated on the microscope in a microcuvette containing and Ca2+-free solutions flowing into the chamber and over the cells.

**Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression.** Wen-hong Li‡, Juan Llopis*, Michael Whitney‡, Gregor Zlokarnik & Roger Y. Tsien*†‡

*Departments of a Pharmacology and b Chemistry & Biochemistry, and a Howard Hughes Medical Institute, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0647, USA

b Aurora Biosciences Corporation, 11101 Torreyana Road, San Diego, California 92121, USA

Inositol 1,4,5-trisphosphate (InsP3) releases calcium from intracellular stores and triggers complex waves and oscillations in levels of cytosolic free calcium. To determine which longer-term responses are controlled by oscillations in InsP3 and cytosolic free calcium, it would be useful to deliver exogenous InsP3, under spatial and temporal control, into populations of unperfused cells. Here we report the 15-step synthesis of a

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membrane-permeant, caged InsP\(_3\) derivative from myo-inositol.

This derivative diffused into intact cells and was hydrolysed to produce a caged, metabolically stable InsP\(_3\) derivative. This latter derivative accumulated in the cytosol at concentrations of hundreds of micromolar, without activating the InsP\(_3\) receptor. Ultraviolet illumination uncaged an InsP\(_3\) analogue nearly as potent as real InsP\(_3\), and generated spikes of cytosolic free calcium, and stimulated gene expression via the nuclear factor of activated T cells\(^{2-7}\). The same total amount of InsP\(_3\) analogue elicited much more gene expression when released by repetitive flashes at 1-minute intervals than when released at 0.5- or \(\geq\)2-minute intervals, as a single pulse, or as a slow sustained plateau.

Thus, oscillations in cytosolic free calcium levels at roughly 1-minute intervals, as a single pulse, or as a slow sustained plateau, are driven by \(\text{InsP}_3\) and can be modulated by various stimuli. The physiological functions of oscillations in cytosolic free calcium ([Ca\(^{2+}\)]\(_c\)) levels and the existence of \(\text{InsP}_3\) oscillations remain controversial\(^{1-4}\). Stimulation with natural agonists does not allow dissection of the relative contributions of oscillation amplitude, frequency, number of pulses, and branching transduction pathways involving, for example, receptor tyrosine kinase activity or diacylglycerol production. It would be preferable to impose oscillations of various frequencies and a steady non-oscillatory elevation, and to compare their efficacies in triggering downstream events such as secretion or gene activation. One could drive [Ca\(^{2+}\)]\(_c\) signals with either \(\text{InsP}_3\) or extracellular Ca\(^{2+}\); the latter strategy\(^8\) also requires elevating the permeability of the plasma membrane to Ca\(^{2+}\). We chose to use \(\text{InsP}_3\) pulses because they mimic the natural cycle of calcium release from internal stores, work

![Figure 1](image_url) 

**Figure 1** Mode of action of the caged, membrane-permeant InsP\(_3\) derivative cmInsP\(_3\)/PM (compound 1), the trapped caged molecule (cmInsP\(_3\) (cmIP\(_3\)), compound 2), and 2,3-methoxymethylene-InsP\(_3\) (mInsP\(_3\), compound 3) released by photolysis. PM, propionyloxymethyl group; DMNB, 4,5-dimethoxy-2-nitrobenzyl group.

![Figure 2](image_url) 

**Figure 2** Synthesis and biochemical characterization of cmInsP\(_3\)/PM and its reaction products. A, Chiral synthesis of cmInsP\(_3\)/PM, starting from myo-inositol. Bz, benzoyl; Camp, \(S\)-camphanyli. Reagents: a, 2-methoxypropene in acidic dimethylformamide (DMF), followed by chromatography and crystallization\(^{10}\); b, Bu\(_2\)SnO, toluene azotrop, 63% yield; c, DMNB bromide, CsF, DMF; d, \(S\)-(\(-\)camphanic acid chloride, triethylamine, \(4\)-dimethylaminopyridine, then silica gel chromatography and crystallization to separate the diastereomeric camphanates, 42% of the desired isomer; e, \(K_2\)CO\(_3\), methanol/CH\(_2\)Cl\(_2\); f, BzCl, pyridine, 80%; g, HSCH\(_2\)CH\(_2\)OH, BF\(_3\)/Et\(_2\)O; h, BzCl, pyridine, 76%; i, HSCH\(_2\)OH, BF\(_3\)/Et\(_2\)O, 80%; j, \(K_2\)CO\(_3\), methanol/CH\(_2\)Cl\(_2\), 52%; l, [NCCH\(_2\)CH\(_2\)O]\(_2\)PN(i-Pr\(2\), tetrazole; m, \(t\)-butyl hydroperoxide, CH\(_2\)Cl\(_2\), 79%; n, NH\(_3\), aqueous methanol; o, Et\(_3\)COOC\(_2\)H\(_5\), (i-Pr\(_2\))_\(_2\)NEt, CH\(_2\)CN, 12% for steps n \(\rightarrow\) o. B, Binding to the InsP\(_3\)R, measured as equivalent picomol of genuine InsP\(_3\) (IP\(_3\)), as a function of duration of ultraviolet-light-mediated photolysis of 8-pmol aliquots of cmInsP\(_3\) (cmIP\(_3\)) in vitro. The smooth curve and dashed line show the fitted exponential progress curve (see Methods) and its asymptote, respectively.

at normal extracellular calcium levels, incorporate the complex relationship between InsP₃ and [Ca²⁺], and may be useful for many other studies.

Standard techniques for delivering the highly charged InsP₃ molecule across cell membranes, such as membrane permeabilization, microinjection, or patch clamping, would not allow reliable measurement of subsequent complex events, such as gene expression, in enough cells. To achieve precise temporal control without disrupting the plasma membrane, we needed a membrane-permeant derivative of caged InsP₃, such as cmInsP₃/PM (compound I in Fig. 1). All three phosphates of this compound were esterified with propionylxymethyl (PM) groups, which hydrolyse in the cytoplasm to produce cmInsP₃ (compound 2 in Fig. 1). Free 2- and 3-hydroxyl groups are not obligatory for binding to the InsP₃ receptor (InsP₃R), so we masked these groups with a methoxymethylene group, which is relatively small and should not interfere with binding to the InsP₃R. Blockage of the 3-hydroxyl should prevent the released InsP₃ analogue from being phosphorylated by InsP₃ 3-kinase to inositol 1,3,4,5-tetrakisphosphate. The 6-hydroxyl of InsP₃ interacts specifically with the InsP₃R and is essential for release of calcium, yet is difficult to regenerate from a permeant ester, perhaps because the bulky flanking 1- and 5-phosphate groups hinder the hydrolysis. Therefore we protected the 6-hydroxyl with a photolabile caging group, a 4,5-dimethoxy-2-nitrobenzyl (DMNB) ether. Ultraviolet-mediated photolysis of the DMNB group should be independent of steric shielding by the flanking phosphates and should abruptly switch on biological activity through formation of compound 3 (2,3-methoxymethylene-InsP₃, or mInsP₃). Also, in the absence of ultraviolet light, the ether linkage should be more resistant to metabolism or hydrolysis than the phosphodiester linkage previously used to cage InsP₃.

CmInsP₃ was synthesized from myo-inositol in 14 steps, then esterified to produce cmInsP₃/PM (Fig. 2A). Binding of cmInsP₃ to the InsP₃R (assayed by displacement of H-labelled InsP₃) was negligible (<0.5% of binding of real InsP₃). However, ultraviolet illumination of cmInsP₃ progressively released material that bound tightly to the InsP₃R (Fig. 2B). The product of exhaustive photolysis bound to the InsP₃R equivalently to 75% of an equimolar amount of real InsP₃. Assuming that mInsP₃ was formed at 100% chemical yield, it is 75% as potent as InsP₃, making mInsP₃ the most potent synthetic InsP₃ analogue known. The quantum yield of photolysis was 0.09 ± 0.013. At 365 nm, the uncaging cross-section of cmInsP₃, 450 M⁻² cm⁻¹, slightly exceeded the 325 M⁻² cm⁻¹ reported for an earlier caged InsP₃, a P₄(5)-1-(2-nitrophenyl)ethyl ester.

Incubation of 1321N1 astrocytoma cells with 2 μM extracellular cmInsP₃/PM for 5, 30 and 60 min resulted in 33 ± 5, 201 ± 9 and 260 ± 10 pmol (mean ± s.e.m.), respectively, of trapped cmInsP₃/PM per million cells. The final values were equivalent to intracellular concentrations of hundreds of micromolar. The ability of cmInsP₃ to accumulate to a much higher concentration inside the cells than the concentration of cmInsP₃/PM outside the cells confirmed that cmInsP₃ was being trapped by irreversible ester hydrolysis, and implied that unphotolysed cmInsP₃ must be quite stable inside living cells. The trapped cmInsP₃ had no effect on [Ca²⁺], even after prolonged incubation, as monitored by the calcium indicator Fluo-3. Subsequent brief exposure of the cells to ultraviolet light (345–355 nm) caused a sudden increase in [Ca²⁺] levels whether extracellular calcium was present or not (Fig. 3a,b), showing that calcium was released from internal stores. Ultraviolet illumination was ineffective if applied less than two minutes after application of cmInsP₃/PM, as would be expected from the time required for the ester to cross membranes and release cmInsP₃. We routinely allowed ≥30 min to ensure complete de-esterification of cmInsP₃/PM after washing away excess ester. Once cmInsP₃ was loaded into cells, it remained well trapped and non-metabolized. At room temperature, an additional incubation time of 8 h in the dark had almost no effect on the size of the subsequent flash-induced [Ca²⁺] transient. Incubation for 8 h at 37°C did weaken the response, but the amplitude could be restored by a roughly threefold increase in exposure to ultraviolet light, indicating that about one-third of the cmInsP₃ remained. Other control experiments with the same or more than ten times stronger ultraviolet light, in the absence or presence of 100 μM 4,5-dimethoxy-2-nitrobenzyl methyl ether as a control for DMNB photolysis byproducts gave no detectable rise in [Ca²⁺] levels, either in normal calcium-containing or in calcium-free medium, showing that the response was specific to cmInsP₃. CmInsP₃/PM behaved similarly in other cell lines, including HeLa cells, human embryonic kidney 293 cells, REF-52 fibroblasts, T84 colonic epithelial cells, RBL-2H3 rat basophilic leukaemia cells (see below), and P388D1 macrophage-like cells, so its biological efficacy seems to be fairly general, at least in cultured mammalian cells.

[Ca²⁺] signals are essential for the activation of lymphoid cells, a process involving gene expression, differentiation and proliferation. Non-invasive loading of cmInsP₃/PM into cells allowed assessment of the effect of different temporal patterns of InsP₃R activation on [Ca²⁺] signals and gene expression. We used activation of the NF-AT (nuclear factor of activated T cells) transcription factor complex in RBL-2H3 cells as a model system. Increased [Ca²⁺] stimulates calcineurin to dephosphorylate pre-existing cytosolic NF-AT proteins (NF-ATc), which then migrate into the nucleus, bind to nuclear proteins and NF-AT-response elements, and activate downstream genes. Although NF-AT was first characterized in lymphocytes as a crucial element in interleukin-2 gene expression, it is widespread in lymphoid cells, including RBL cells. We assayed NF-AT-driven gene expression at the level of single cells, using a stably transfected reporter construct in which a trimer of NF-AT-response elements drove expression of the bacterial enzyme β-lactamase. β-Lactamase activity can be assayed with a newly developed membrane-permeant fluorescent substrate, which becomes trapped in individual live cells. In the absence of β-lactamase, the cells fluoresce at 520 nm, whereas expression of the
**Figure 4** Expression of the β-lactamase reporter gene induced by uncaged cmlnSP3/PM. **a,** Unstimulated cells. **b,** Cells stimulated by 1 μM ionomycin for 20 min at 37°C. **c, d,** Cells loaded with 10 μM cmlnSP3/PM were illuminated with four pulses each of ultraviolet light of 0.3, 0.6, 1, and 1.4 s duration, spaced 1 min (**c**) or 3 min (**d**) apart. The cells were then incubated for 3.5 h at 37°C, and then stained for β-lactamase gene expression. The ratio of 420–460 nm to 512–558 nm emissions while exciting at 392–408 nm is displayed in false colours calibrated by the colour scale at the right, which also indicates the threshold ratio (0.35) for a cell to be scored as positive for reporter gene expression.

**Figure 5** [Ca2+], levels measured by Fluo-3 fluorescence and NF-AT-driven β-lactamase gene expression evoked by different temporal patterns of photolysing the same total amount of cmlnSP3 in RBL cells. **a–g,** The time and Fluo-3 intensity scales at the upper left apply to all traces. **a,** 3.5 × 10⁻⁶ einstein cm⁻² s⁻¹ or 11.4 mW cm⁻² was delivered for 10.4 s where indicated. **b–e,** 12 Pulses of the same intensity were delivered in three groups of four flashes, each of 0.3, 0.8, and 1.5 s duration, spaced 30 s (**b**), 1 min (**c**), 2 min (**d**), and 8 min (**e**) apart. **f,** Continuous illumination with (2.42, 2.7, or 3.1) × 10⁻⁶ einstein cm⁻² s⁻¹, each for 7 min, represented by dotted, dashed, and solid lines, respectively. **g,** 50 ng ml⁻¹ DNP-BSA was added at the point indicated by the arrow. Traces in **f, g** are from representative single cells, whereas traces in **a–e** are averages of 25 cells because the responses were much more homogeneous and synchronous. **g,** The average frequency of spikes of amplitude ΔF/F₀ = 0.5 was 2.09, 0.66, and 0.39 min⁻¹ at the left, centre and right, respectively. **h,** NF-AT-driven β-lactamase gene expression. Percentage of cells is shown with suprathreshold β-lactamase activity resulting from protocols a–g.
enzyme cleaves the substrate and changes the fluorescence maximum to 447 nm. As this new reporter gene assay leaves the cells fully viable, we could use fluorescence-activated cell sorting to isolate a subclone of RBL cells in which NF-AT-driven gene expression was maximally responsive to sustained elevation of [Ca^{2+}]. Levels of Ca^{2+} spikes. The single burst of InsP3 or excessively frequent oscillations of InsP3 (Fig. 5c) were much better than continuous dribbling of the same total amount of InsP3 (Fig. 5f) for producing big and reliable [Ca^{2+}] spikes, which in turn optimally activated at least one prototypic calcium-responsive transcription factor (see also ref. 8). Other genes and cell functions dependent on InsP3 and [Ca^{2+}] should also be investigated by this new, convenient method to activate the InsP3R under spatiotemporal control in large populations of fully intact cells.

Methods

Synthesis of cmlnS3 and cmlnS3/PM. See Fig. 2A. Fast atom bombardment mass spectroscopy showed an exact mass for compound 1 + Ca^{2+} of 1,305.1481 compared with 1,305.1444 calculated for C_{41}H_{62}O_{32}NP_{3}. Measurement of loading efficiency of cmlnS3/PM. Determination of the photoysis quantum yield of cmlnS3 and affinity of binding of mlnS3 to the InsP3R.

Determination of the photoysis quantum yield of cmlnS3 and affinity of binding of mlnS3 to the InsP3R. The quantum yield, of the photoysis was calculated from the exponential progress curve the extinction coefficient (5 x 10^{-2} cm^{-2} mol^{-1}) of cmlnS3 at 365 nm, and the ultraviolet intensity (1.41 x 10^{-8} einstein cm^{-2} s^{-1}) measured by ferroxyline actinometry.

Determination of loading efficiency of cmlnS3/PM. Confluent monolayers of astrocytoma cells in 35-mm tissue culture dishes were incubated with 2 μM cmlnS3/PM for the indicated time. After washing and 30 min incubation, the cell medium was aspirated and the cells were quenched with 0.5 ml ice-cold perchloric acid containing 2 mM EDTA and 20 mM HEPES. The cmlnS3 was then thoroughly photolyzed at 0°C with 365 nm ultraviolet light. The samples were neutralized with KOH, centrifuged to remove KClO4, and assayed for InsP3 normalized to the number of cells. Experiments were done in duplicate on separate dishes of cells.

Monitoring cytosolic Ca^{2+} transients produced by uncaging cmlnS3. 121N1 astrocytoma cells were loaded with cmlnS3/PM (2 μM) and Fluo-3/AM (2 μM) in HBS containing 0.05% Pluronic F-127 for 1 h at room temperature. Cells were then washed and incubated for another 30 min. Fluo-3 intensity (F, 480 ± 15 nm excitation, 535 ± 22.5 nm emission) was monitored every 7 s by digital-imaging microscopy and was plotted, after background subtraction, as a ratio against the Fluo-3 intensity at the resting calcium level (F0). Ultraviolet pulses for the experiments of Fig. 3 were from a 150 W xenon arc, attenuated by a neutral density filter of 4% nominal transmission, at 0°C with 365 nm ultraviolet light. The samples were neutralized with KOH, centrifuged to remove KClO4, and assayed for InsP3 normalized to the number of cells. Experiments were done in duplicate on separate dishes of cells.

Generation of an RBL cell line stably incorporating the NF-AT-β-lactamase transcriptional reporter. The pZeoN–NF-AT–BLA vector was constructed by cloning a trimer of NF-AT–response-element promoters upstream of a cyttoplasmic form of β-lactamase. This construct was made using the pZeoSv (Invitrogen) expression vector minus its SV40 promoter. RBL-2H3 cells were transfected by electroporation with pZeo–NF-AT–BLA DNA and selected in RPMI-1640 medium with 10% fetal bovine serum and 250 μg/ml zeocin for 2 weeks. The stably transfected population of RBL cells was
subconed by stimulating with 1 μM ionomycin for 3 h, vital staining with the membrane-permeant β-lactamase substrate CCF2/AM, and fluorescence-activated cell sorting. The best clone gave 95–100% β-lactamase-positive cells with such stimulation and was used for all the subsequent studies on RBL cells.

Uncaging cmlnP2 and measurement of reporter gene expression. The RBL cells were loaded with 10 μM cmlnP2/PM for 30 min (Fig. 4) or with 6 μM ester for 1 h (Fig. 5), and were then incubated in ester-free medium for 0.5 h at room temperature. Uncaging of cmlnP2 was performed at 37°C in culture medium in a sealed thermostated perfusion chamber (Bioprocess, Butler, PA) with the same shuttered mercury lamp as for the [Ca²⁺]c measurement. The cells were left for 3.5 h at 37°C, loaded for 30 min at room temperature with 5 μM of the β-lactamase substrate CCF2/AM in PBS containing 0.05% Pluronic F-127, and finally washed and incubated for 30 min at room temperature. β-Lactamase expression was scored by exciting at 392–408 nm wavelength and viewing the ratio of 420–460 to 512–558 nm emissions. A charge-coupled-device camera (Photometrics) and ratio-imaging software (Metafluor, Universal Imaging) were used to obtain the data shown in Fig. 4 and to score borderline cells for Fig. 5. Cells whose 420–460 nm signal was above 1.5 times the background were scored as positive.

Control stimuli for gene expression. For exposures to ultraviolet light in the absence of cmlnP2/PM, we used the uncaging protocols of Fig. 5b or Fig. 5c. Ionomycin (1 μM) treatments at 37°C were terminated by washing with fresh medium containing 5 mM bicine bovine serum albumin to sequester the ionophore. The cells were then incubated with 1.3 mM Ca²⁺ (Fig. 4b) or 3 mM EGTA. In the latter case, 15 and 30 min incubations with ionophore activated β-lactamase in 28 ± 5 and 65 ± 9% of the cells, respectively. To stimulate with antigen, the RBL cells were passively sensitized for 12 h with monoclonal murine IgE antibodies against dinitrophenyl (DNP) (Sigma). The cells were washed five times with fresh medium and incubated at 37°C for 45 min before antigen stimulation. Dinitrophenylated bovine serum albumin (DNP-BSA, Calbiochem) was added at a final concentration of 50 or 500 ng/ml. 50 ng/ml DNP-BSA caused the highest degree (in both amplitude and duration) of intracellular calcium mobilization, whereas the calcium transients in individual cells were heterogeneous. 500 ng/ml DNP-BSA caused negligible mobilization of intracellular calcium and activated β-lactamase in only 2.1 ± 0.9% of cells.

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Correspondence and requests for materials should be addressed to R.Y.T.

NMR structure and mutagenesis of the FADD (Mort1) death-effector domain

Matthias Eberstadt, Baohua Huang, Zehan Chen, Robert P. Meadows, Shi-Chung Ng, Lixin Zheng*, Michael J. Lenardo* & Stephen W. Fesik

Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064, USA
* Laboratory of Immunology, National Institutes of Health, Bethesda, Maryland 20892, USA

When activated, membrane-bound receptors for Fas and tumour-necrosis factor initiate programmed cell death by recruiting the death domain of the adaptor protein FADD (Mort1; ref. 2) to the membrane. FADD then activates caspase 8 (ref. 3) (also known as FLICE or MACH) through an interaction between the death-effector domains of FADD and caspase 8. This ultimately leads to the apoptotic response. Death-effector domains and homologous protein modules known as caspase-recruitment domains have been found in several proteins and are important regulators of caspase (FLICE) activity and of apoptosis. Here we describe the solution structure of a soluble, biologically active mutant of the FADD death-effector domain. The structure consists of six anti-parallel, amphipathic α-helices and resembles the overall fold of the death domains of Fas and p75 (ref. 16). Despite this structural similarity, mutations that inhibit protein–protein interactions involving the Fas death domain have no effect introduced into the FADD death-effector domain. Instead, a hydrophobic region of the FADD death-effector domain that is not present in the death domains is vital for binding to FLICE and for apoptotic activity.

As neither the wild-type death-effector domain (DED) of FADD nor full-length FADD could be obtained in a form that was suitable for study by nuclear magnetic resonance (NMR), we prepared several site-directed mutants and screened them for their ability to be expressed as a soluble protein, induce apoptosis and bind to FLICE. On the basis of its high solubility in a monomeric form (0.7 mM) and wild-type-like biological activities (Fig. 1), we chose the Phe 25→Tyr (F25Y) mutant protein for structure determination.

The structure of the F25Y FADD DED is well defined by the 1,102-atom NMR structure and c in which the peptide amide protons are assigned (Fig. 2). The structure consists of six antiparallel, amphipathic α-helices (Fig. 2b). The α-helices are

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