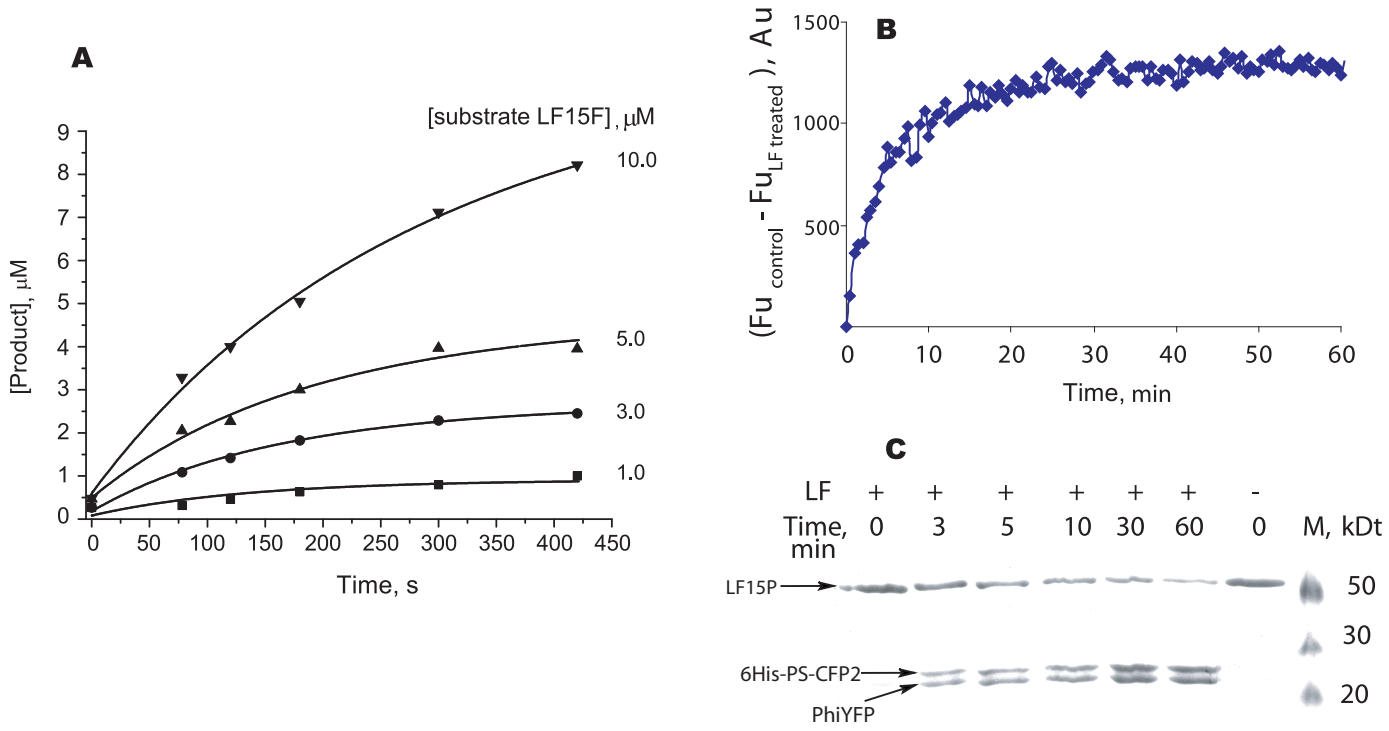
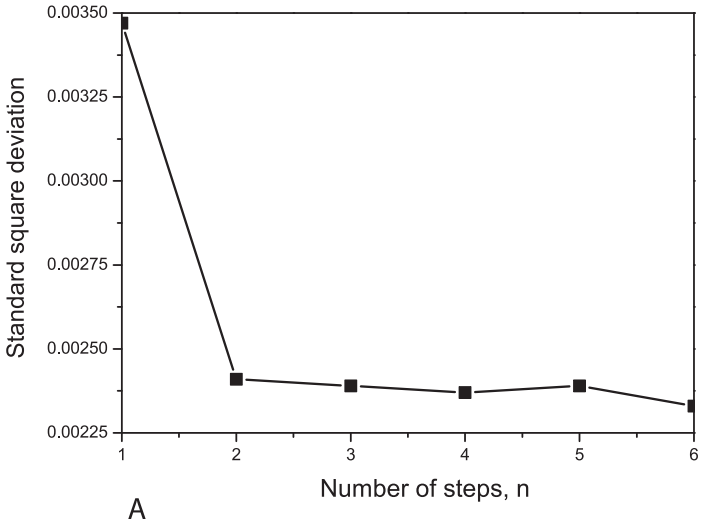
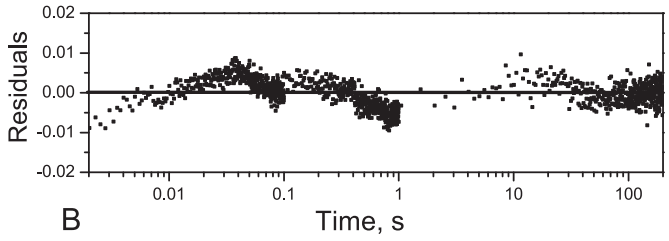


Supplementary Fig. 1. Time course for LF-mediated hydrolysis of fluorescent substrates under steady-state conditions. (A) cleavage of LF15F analyzed by reverse-phased HPLC. Enzyme concentration was 50 nM. Substrate concentrations are indicated on the figure. Product concentrations were calculated by integrating chromatogram peak areas, using Waters 501 software. Dotted lines represent experimental data, smooth lines display fitting to the Michaelis-Menten equation.The resulting constants are presented in Table 3 (steady-state K_m and k_{cat} for LF15F substrate); (B) and (C) cleavage of LF15P FRET fusion protein substrate, monitored by (B) fluorometr, measuring the difference in FRET signal between intact and cleaved, and (C) by gel electrophoresis. Enzyme and substrate concentrations were 50 nM and 5 μM, respectively.

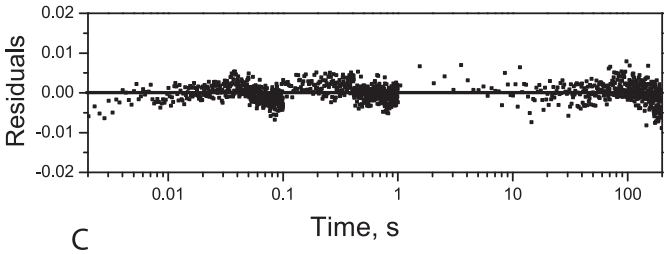
Supplemental figure 2



A

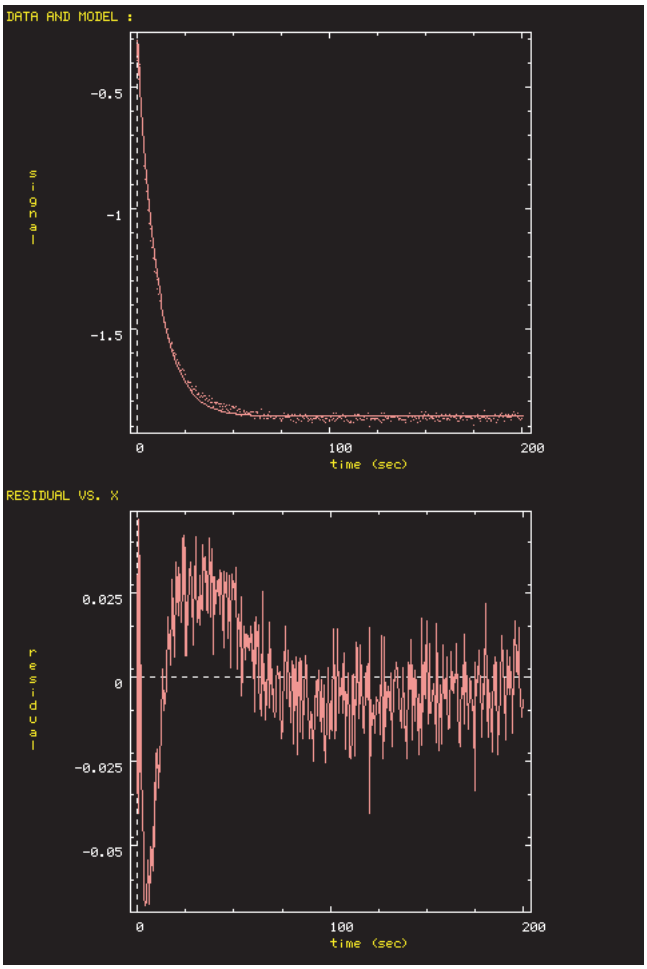


B



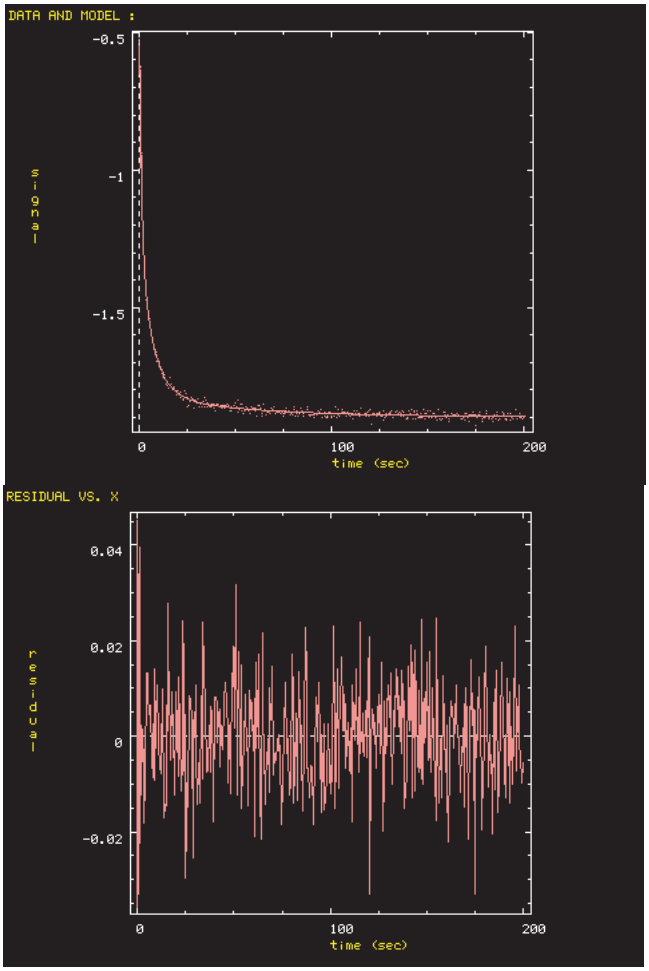
C

Mechanism: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{\cdot}S) \xrightarrow{k_2} (E{\cdot}P) \overset{K_P}{\rightleftharpoons} E + P)$



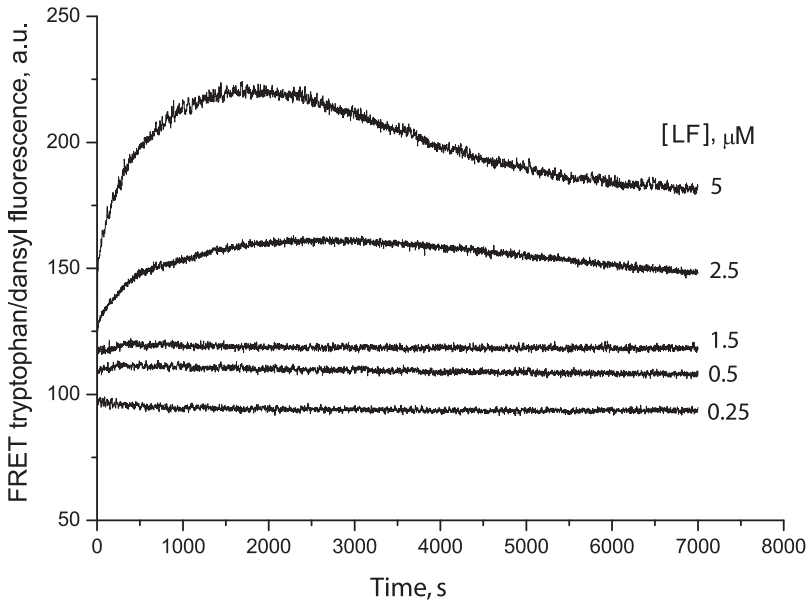
D

Mechanism: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{\cdot}S)_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E{\cdot}S)_2 \xrightarrow{k_3} (E{\cdot}P) \overset{K_P}{\rightleftharpoons} E + P)$



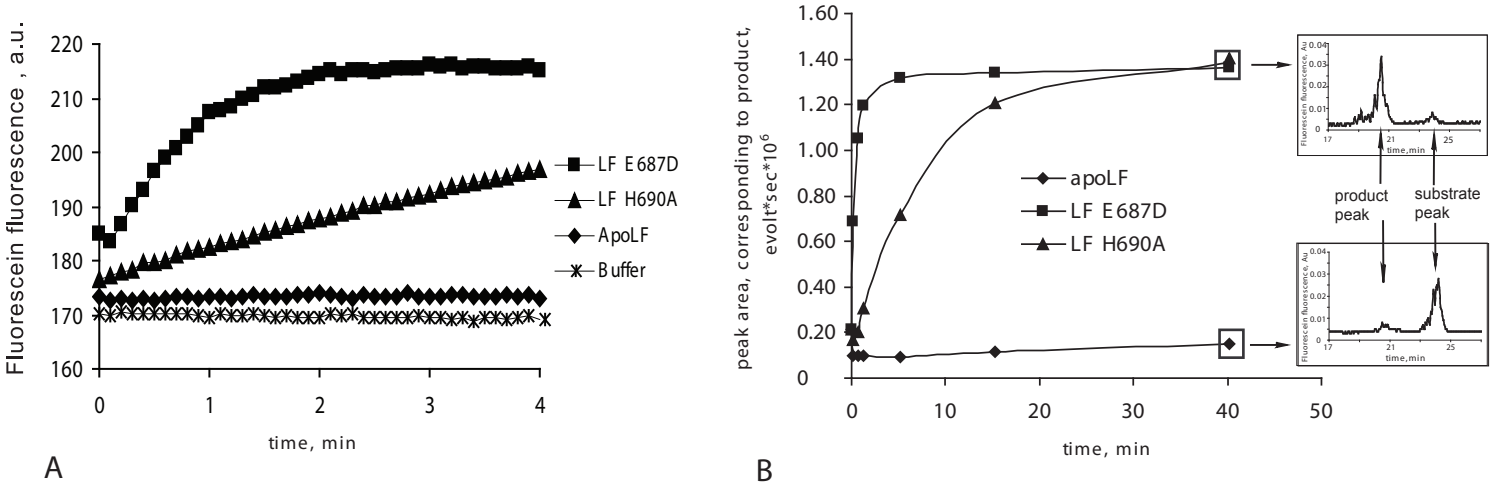
E

Supplementary Fig. 2. Statistical scree test employed for validation of proposed kinetic mechanisms. (A) The "scree-test" for LF/LF15F interaction represents the dependence of standard square deviations of the data residuals after fitting to an *n*-step binding model on *n* (scheme 1 (Fig 2A)). The number of steps corresponding to the beginning of the shallow slope (scree) region is considered minimal for adequately describing the binding. (B) and (C) represent the data residuals for single- (B) or two-step (C) schemes of the substrate binding. LF and LF15F were taken in a 1:1 molar ratio (5 μM). It can be clearly seen that the smooth decrease in the standard deviations levels after two steps, indicating that the two-step kinetic scheme is indeed the minimal one for substrate binding. The scree test was applied for every step of each kinetic scheme. Figures D & E demonstrate examples of "bad" and "good" fitting to different kinetic mechanisms: D, result of Dynafit analysis for single-step substrate binding, E, fitting to the two-step binding scheme. Upper boxes demonstrate experimental data (dotted lines) and resulting fitting curves (smooth lines), lower boxes show square residuals for these curves.



Supplementary Fig. 3. Time course for hydrolysis of dansylated S20D substrate by LF. Data collected reflects changes in FRET signal between dansyl label and enzyme Trp residues. The assay was performed using Varian Cary Eclips spectrofluorometer and designed to collect the hydrolysis data outside the timing constraints of the of stopped-flow device (for the initial stopped-flow curves see Fig. 1D). Enzyme and substrate were mixed in the same ratio as in the stopped-flow experiment. Substrate concentration was 5 μM. Enzyme concentrations are indicated in the figure.

Supplementary fig 4



Supplementary Fig. 4. (A) Kinetics for interaction of LF mutant forms and LF apoenzyme with LF15F substrate. Concentration of substrate and enzymes were 5 μM. The assay was performed using Varian Cary Eclips spectrofluorometer and designed to compare the proteolytic activity of completely inactive apo-LF devoid of any divalent metal ions, and LF E687D and H690A mutants. Designations for respective reactions are shown in the picture. Data collected for control reaction proceeded in the absence of enzyme is designated as "buffer". (B) HPLC-based analysis of LF15F cleavage with apo-LF and mutant LF species. Concentration of substrate and enzymes were 5 μM. Designations for respective reactions are shown in the picture. Arrows point from the respective curves to drawing inserts and indicate the state of LF15F at the respective reaction point (each shown enclosed in a square). Arrows pointing at "product peak" and "substrate peak" indicate chromatographic mobility of input substrate and cleavage product calculated basing on the previous reactions carried out using wild-type LF.

Supplementary Table I Oligonucleotides and peptide sequences, used in this study

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Primer name	Primer sequence 5'-3'	Peptide sequence at the N-terminus of pIII formed by a primer pair (where applicable)
FdMycFor	TC TAT ATA ACT TAC AGT GCA CAG ACT GAG CAG AAG CTT ATC TCC GAA GAG GAC CTG GGC GGT TCT	AQTEQKLISEEDLGGSGRRKKVYPYPMELE
$^{+}$SuPhageRev	TC TCT TAT CTC GAG TTC CAT TGG GTA CGG ATA CAC TTT CTT GCG ACG GCC AGA ACC GCC CAG GTC CTC	
FdMycFor		MAQTEQKLISEEDLGGSGRLE
$^{-}$SuPhageRev	TC TCT TAT CTC GAG ACG GCC AGA ACC GCC CAG GTC CTC	
FdMycFor		ALTEQKLISEEDLGGSGNNNNNNNNSLE
RandFdXho	ATC TCT TAT CTC GAG AGA VNN VNN VNN VNN VNN VNN VNN GCC AGA ACC GCC CAG GTC CTC	
2MycAscFor	T TAT TCT AAG CTT ATC TCC GAA GAG GAC TTG AGC GGT TCT GGC GAG CAA AAA CTG ATT TCT GAG GA	AQTEQKLISEEDLSGSGEQKLISEEDLGSGAPPSSLE§
2MycAscRev	TTA TAT ATC TCG AGG GAA GAC GGA GGC GCG CCA GAA CCT AAA TCT TCC TCA GAA ATC AGT TTT TGC T	
$^{+}$SuPhageRev		SGSDLGGSGRRKKVYPYPMELE§
LibAscFor		
$^{-}$SuPhageRev		SGSDLGGSGRLE§
LibAscFor	CTT ATA TAT TCT ATT ATA GGC GCG CCT TCT GGT TCT GAC CTG GGC GGT TCT GGC	
LibAscFor		SGSDLGGSGRX[T/S/F]RR][V/I][T/S/N/K/P/H/I]XXXXXLE§
2LibRev	CTA TAT ATC TCG AG NNN NNN NNN NNN NNN DDW AAY GCG ACG GRW DWH ACG AGC GCC AGA ACC GCC CAG GTC	
LFfor	ACA AGA GGA TCC GGC GGG C GG TCA TGG TGA TGT AGG T	
LFrev	CTA A TT GTC GAC TTA TGA GTT AAT AAT GAA CTT AAT C	
Peptide name	**Peptide sequence**	**Remarks**
LF15F/LF15D	Flu/Dans – QRRKKVYPYPME	LF15F is labeled by fuorescein (Flu), whereas LF15D is labeled with dansyl (Dans)
S20D	Dans – RDIRRITLFSLH	-
S40F	Flu – RDFRRIIAERYL	-
S82F	Flu – RIIRRVNSSLPL	-

*All the primer sequences are shown once, if repeated with respect to a peptide sequence, only primer name is shown

§ Peptides shown assuming that the dual c-myc peptide (2mycAscFor+2MycAscRev) is already present at the tip of pIII

Supplementary Table II. Structure of LF substrates selected from the 8-amino acid randomized substrate phage library.

	$P_{10}P_9P_8P_7P_6$	$P_5P_4P_3$	P_2	$P_1P_1'P_2'P_3'P_4'$
consensus	+	+ x x	h	x↓h
substrates				
LF15	r r	k k v	y	p↓y p m e
1	*S G* H	P R V	Y	L N H *S E*
2	V F Q V T	S R V	*S*	*L E E T V*
3	W P P	L R V	S	T *S L E E*
4	A R A V F	K R A	*S*	*L E E T V*
5	T P L S	R R P	T	*S L E E T*
6	I S S	R R A	L	I *S L E E*
7	F Y F	R R V	S	R *S L E E*
8	F F R F F	R R V	*S*	*L E E T V*
9	Y R M T	R R I	T	*S L E E T*
Sub library structure	R X T/S/F	R R V/I	T/S/N/K/P/H/I	X X X X X

Positions, surrounding the scissile bond are numbered as P8 to P4' from N- to C-terminus of the substrate, the scissile bond is the P1-P1' one. Substrates were selected as cleavable by LF after screening of the first-iteration library. To prepare the 2nd-iteration library (sub library), conserved amino acids selected after screening of the 8-aa library were fixed, other positions were either partially (P7, P6, P3, and P2) or fully (P1 to P4') randomized. LF15 is the substrate efficiently cleaved by LF and isolated previously from synthetic peptide library (7). In each substrate invariant amino acids are underlined.