Supplementary Material

A second set of cross-correlation experiments was performed with Cy5-labeled β C His_6 proteasome and Oregon-Green 488-labeled insulin B chain to extract the dissociation constant of the theoretical single-substrate binding state from the steady-state equilibrium. The auto- and cross-correlation curves are depicted in Fig. S1. The corresponding parameters were derived as described previously (Table S2). The cross-correlation curve documents that labeled insulin B chain associates with labeled proteasome, but does not discriminate between single- and multiple-substrate binding states. However, the cross-correlation product exhibited a lower concentration than in the previous experiment, because the proteasome concentration had to be decreased due to detection limits. Since the cross-correlation volumes in the two studies were equally sized, the diffusion times of the substrate-bound proteasomes were comparable (~ 370 µs). According to a hypothetical one-substrate binding model, the dissociation constant could be calculated (K_D = 1.1 µM). As this dissociation constant is more than fourfold lower than concluded from the previous cross-correlation experiment, binary substrate-binding events contribute to the steady-state equilibrium. An additional effect of the proteasomal Cy5-label on the dissociation constant cannot be excluded.

Fluorescent labeling of the proteasome

Concentrated His-tagged proteasomes were diluted 1 : 1 in 100 mM Na_2CO_3, pH 9.0 and incubated for 30 min with a 60-fold molar excess of Cy5-NHS ester at 25°C in the dark. The labeled proteasome was immediately dialyzed overnight against HEPES buffer to remove unreacted dye. Finally, the labeled proteasome was concentrated in Centricons (MWCO: 30 kDa) and separated on a Superose 6 column as described before. The identity and labeling was verified by SDS-PAGE. The labeling ratio was determined photometrically.

Cross-correlation experiment with labeled proteasome

The cross-correlation experiment was described previously, except that the pinhole adjustment and confocal volume determination in channel 1 was performed with Cy5 (D = 2.19*10^{-10} m^2/s (Carl Zeiss, Jena)). The diffusion times of the proteasome-bound substrates in the first study were compared with the diffusion time of Cy5-labeled β C His_6 proteasome (20 - 50 nM) in the presence of Oregon-Green 488-labeled insulin B chain (20 – 50 nM).

Concentration determination in cross-correlation

The confocal volume in the green and red channel is determined by the corresponding radii

$$V_g = \pi^{3/2} \cdot \omega_{1,g}^2 \cdot \omega_{2,g} \text{ and } V_r = \pi^{3/2} \cdot \omega_{1,r}^2 \cdot \omega_{2,r}. \qquad \text{Eq. 3a,b}$$

The overlapping volume in cross-correlation is created by vectorial addition:

$$V_{gr} = \left(\frac{\pi}{2}\right)^{3/2} \cdot \left(\omega_{1,g}^2 + \omega_{1,r}^2\right) \cdot \sqrt{\omega_{2,g}^2 + \omega_{2,r}^2}\ . \qquad \text{Eq. 4}$$

The particle numbers are derived from the amplitudes G(0) of the two autocorrelation functions (AC,g and AC,r) and the cross-correlation function (CC):

$$G_g(0) = 1 + \frac{1}{N_{AC,g}},\ G_r(0) = 1 + \frac{1}{N_{AC,r}} \text{ and } G_{gr}(0) = 1 + \frac{1}{N_{CC}}. \qquad \text{Eq. 5a,b,c}$$

Thus the ensemble of molecules labeled with green or red dye, respectively, can be described as

$$N_{AC,g} = N_g + N_{gr} = \frac{1}{G_g(0) - 1} \text{ and } N_{AC,r} = N_r + N_{gr} = \frac{1}{G_r(0) - 1}\ . \qquad \text{Eq. 6a,b}$$

The number of molecules labeled with both dyes is proportional to the cross-correlation amplitude $G_{gr}(0)$

$$N_{gr} = \frac{N_{AC,g} \cdot N_{AC,r}}{N_{CC}} = \frac{\left(N_g + N_{gr}\right) \cdot \left(N_r + N_{gr}\right)}{N_{CC}}. \qquad \text{Eq. 7}$$

The number of molecules bearing green dye only (N_g) or red dye only (N_r) can be calculated by rearrangement of Eq. 6a,b. In the first cross-correlation experiment, the crosstalk Q_{gr} of the green emission into the red channel has to be taken into consideration

$$N_g = N_{AC,g} - N_{gr} \text{ and} \qquad \text{Eq. 8a}$$

$$N_r = N_{AC,r} - \left(Q_{gr} \cdot N_{AC,g} + N_{gr}\right) \qquad \text{Eq. 8b}$$

$$N_{gr} = \frac{N_{AC,g} \cdot \left(N_{AC,r} + Q_{gr} \cdot N_{AC,g}\right)}{N_{CC} - Q_{gr}}. \qquad \text{Eq. 8c}$$

The molar concentrations are calculated from the particle numbers in the corresponding confocal volumes:

$$c_x = \frac{N_x}{V_x \cdot A} \text{ with x = g, r or gr,} \qquad \text{Eq. 9}$$

where A denotes Avogadro´s number.

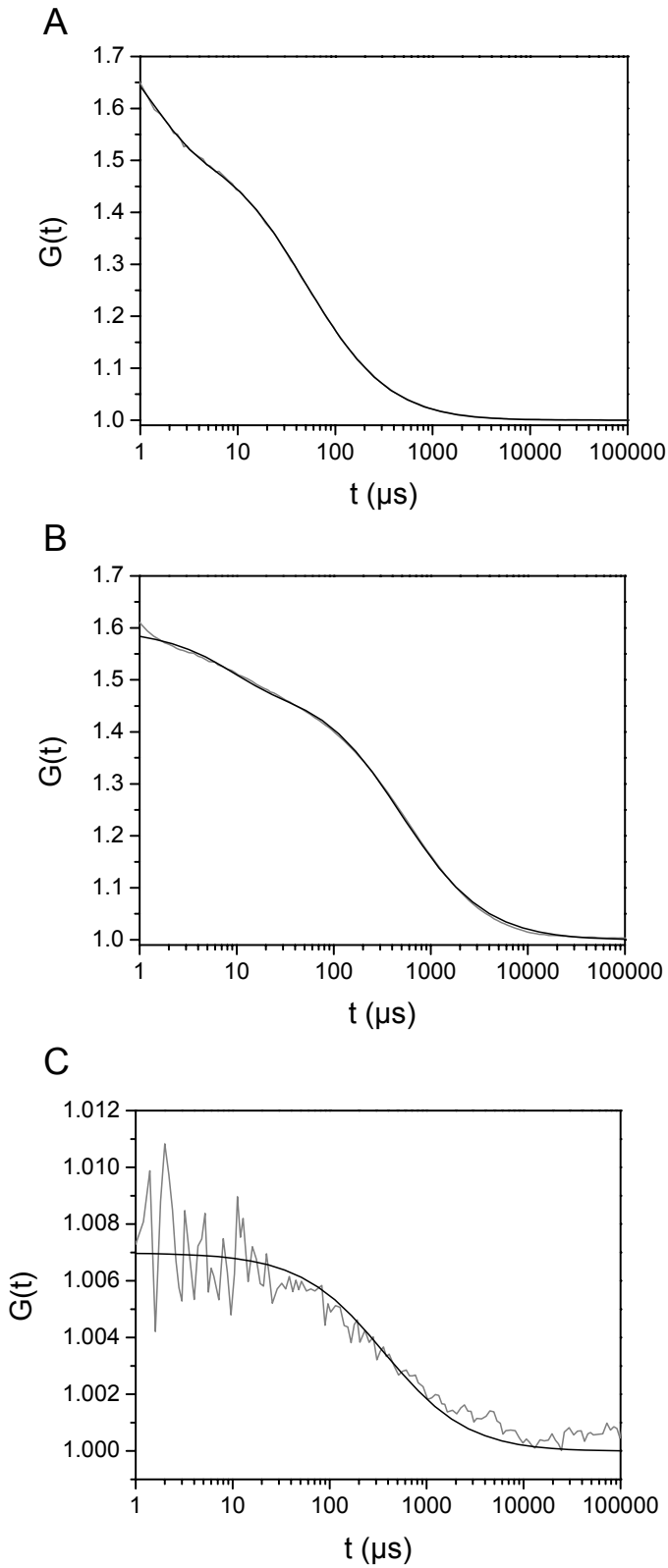
Figure S1

Cross-correlation experiment studying the interactions of green-fluorescent substrate with red-fluorescent proteasomes. Autocorrelation curves (λ_{ex} = 488 nm (A) and λ_{ex} = 633 nm (B)) and cross-correlation curves (C) were monitored with Cy5-labeled β C His_6 proteasome (32 nM) in the presence of Oregon-Green 488-labeled insulin B chain (31 nM) and fitted with one-component models.

	AC 488	AC 633	CC
τ_{dye}/μs	24.9	52.9	-
S	6.3	8.4	7.4
V/fl	0.163	0.467	0.306
G(0)	1.533	1.485	1.006
N	1.8528	2.0388	0.0232
c/nM	18.9	7.25	0.126
τ/μs	49.4 ± 0.2	493.1 ± 5.0	367.5 ± 36.5

Table S2

Data extracted from the cross-correlation experiment of Cy5-labeled proteasome with Oregon-Green 488-labeled insulin B chain. Auto- (AC 488 and AC 633) and cross-correlation curves (CC) were recorded with Cy5-labeled β C His_6 proteasome in the presence of Oregon-Green 488-labeled insulin B chain. Data were derived from the plots as discussed in Materials & Methods.



Hutschenreiter et al., Fig. S1