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Mechanism of the α-complementation reaction of *E. coli* β-galactosidase deduced from fluorescence correlation spectroscopy measurements

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Abstract

The kinetics of the α-complementation reaction of two protein fragments yielding active *E. coli* β-galactosidase was measured using fluorescence correlation spectroscopy (FCS). The association reaction was extremely slow with an apparent association rate k_{app} of 207 M^{-1} s^{-1}. This low association rate can be explained by a fast pre-equilibrium and slow subsequent steps involving at least two dimeric complexes. The subsequent formation of a tetrameric complex is probable and consistent with the experimental data. The complexes comprise two or four subunits, respectively, of the large fragment $(EA)_2$ and in all cases only one small fragment, ED which has been labeled with Cy5. These kinetics have been compared to the association kinetics of ED to inactivated $(EA)_2$. The kinetics were similar to the association with native $(EA)_2$. The data support the observation that lyophilization of $(EA)_2$ in a reducing environment which causes complete loss of enzymatic activity does not interfere with binding.

Keywords: β-Galactosidase; Reaction mechanism; Association kinetics; Fluorescence correlation spectroscopy; CEDIA™

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1. Introduction

The association kinetics of the well-known α-complementation of *E. coli* β-galactosidase [1–5] were evaluated using fluorescence correlation spectroscopy (FCS). β-Galactosidase is formed by the spontaneous association of a large dimeric polypeptide (200 kDa), denoted enzyme acceptor $(EA)_2$, and a small polypeptide (approx. 20 kDa), denoted enzyme donor (ED). EA and ED are enzymatically inactive but spontaneously associate to give enzymatically active β-galactosidase. In CEDIA™ assays the hapten, or analyte, is covalently linked to an ED analog and an analyte-specific antibody is used to inhibit the assembly of enzymatically active β-galactosidase [4–6]. Analyte in a patient's serum competes with the analyte in the analyte–ED analog conjugate for antibody, modulating the amount of active β-galactosidase formed. The signal generated by enzyme substrate is therefore directly proportional to the analyte concentration in the patient's serum.

Further optimization of the CEDIA™ assay system requires insight into the reaction mechanism. Characterization of the association process will help to identify factors which affect the precision of the assay. It is important to know whether an enzymatically inactive $(EA)_2$ variant is incapable of binding ED, or has merely been altered at its active site while retaining binding capacity. To date, it has only been possible to indirectly evaluate the association kinetics at very low ED concentrations by measuring the turnover of β-galactosidase substrate. With such measurements, however, it is not possible to determine whether a lack of substrate turnover is rooted in a lack of subunit association, or a loss of enzyme activity in a correctly assembled form of β-galactosidase. With time-resolved FCS measurements, this constraint has been overcome. A detailed study of association was performed in order to elucidate the reaction mechanism prior to enzymatic conversion of the substrate. The data provide strong evidence for the existence of a fast pre-equilibrium state followed by a comparably slower conversion into a stable complex.

The association of ED-Cy5 with both native and inactivated $(EA)_2$ was measured using FCS. $(EA)_2$ was inactivated in the presence of reducing agents and subsequently lyophylized prior to FCS analysis. It could be shown that the inactivated $(EA)_2$ variant was able to bind ED though enzymatic activity was lost. The dynamics of the binding was similar to that of native $(EA)_2$ to ED-Cy5. In the case of the inactive $(EA)_2$, we conclude that the active catalytic center was converted into an inactive conformation, whereas the binding domain was not significantly affected.

2. Materials and methods

2.1. Substances

A stock solution of native, wild-type $(EA)_2$ (Microgenics) was prepared by dissolving 23 mg in a 300-μl assay buffer (100 mM MOPS, 400 mM NaCl, 10 mM EGTA, 3 mM Mg-acetate, 20 mM Na-azide, 0.005%% Tween 20, pH 6.8) to give a final $(EA)_2$ concentration of 375 μM. Another fraction of $(EA)_2$ was denatured by lyophilization of wild-type $(EA)_2$ in glycerol/DTT buffer. This solution was dialyzed extensively against the assay buffer. The $(EA)_2$ concentrations were determined by the BCA test (Pierce) with native $(EA)_2$ as standard. ED (6.7 μM), labeled with Cy5™ (Microgenics), was diluted in assay buffer.

2.2. Equipment

ConfoCor® (Carl Zeiss, Jena/EVOTEC BioSystems GmbH, Hamburg, Germany) with interference filter 670DF40 (Omega), helium–neon laser (632-nm line, 300 μW, Uniphase), C-APOCHROMAT 40 $\times$ /1.2 objective (Carl Zeiss Jena), pinhole 116 μm. The photon count signal was autocorrelated over 30 or 60 s.

2.3. Data evaluation

Evaluation of the autocorrelation curves was carried out with a Marquardt non-linear least-square fitting routine using the following two-component model corresponding to free and bound ED [12–14]:

$$G(\tau) = \left[1 - T + T\exp\left(\frac{-\tau}{\tau_T}\right)\right] N^{-1}\left[\frac{1-Y}{\left(1+\frac{\tau}{\tau_{\text{free}}}\right)\sqrt{1+\frac{r_0^2}{z_0^2}\frac{\tau}{\tau_{\text{free}}}}} + \frac{Y}{\left(1+\frac{\tau}{\tau_{\text{bound}}}\right)\sqrt{1+\frac{r_0^2}{z_0^2}\frac{\tau}{\tau_{\text{bound}}}}}\right] \quad (1)$$

where T is the average fraction of dye molecules in the triplet state with relaxation time τ_T, N is the total average number of fluorescent molecules in the observation volume, Y is the relative concentration fraction of bound ED ($[ED_{bound}]/[ED_{total}]$), τ_{free} and τ_{bound} define the average time (diffusion time) for detected molecules of free and bound ED, respectively, and where r_0 and z_0 are the lateral and axial distances between the coordinate where the Gaussian emission light distribution adopts the maximum value and the point where the light intensity decreases down to $1/e^2$ of the maximum value (observation volume).

2.4. Reactions

The concentration of ED (5 or 10 nM) was kept constant in a specific series of experiments. The $(EA)_2$ concentration varied from 0.13 to 9.9 μM. All incubations and measurements were carried out at room temperature (22 ± 2°C). The measurement times for each point in the association kinetic curve were 30 s for $(EA)_2$ concentrations of more than 1 μM, and 60 s for $(EA)_2$ concentrations of 0.13–0.66 μM.

2.5. Simulations

Each model (1–5) of possible reaction mechanisms for ED and $(EA)_2$ can be described by a system of ordinary differential equations (ODEs). These ODEs define the changes in concentration with time of the molecular species involved. Since the ODEs turned out to be a rigid system, the Rosenbrock method [10] was employed for its numerical solution. The models with the corresponding ODEs are compiled in Table 2.

3. Results and discussion

The association kinetics of ED (to which the fluorophore Cy5 was attached) and $(EA)_2$ were evaluated using FCS. The results of these association experiments are presented here. Several different reaction mechanisms are proposed which explain for the experimental data with varying degrees of precision.

3.1. Association kinetics

Association experiments were carried out with ED and $(EA)_2$ concentrations close to those used under assay conditions described for the CEDIA™ system. The time course of the association was followed using time-resolved FCS. All association experiments were performed under 'pseudo' first order conditions, with at least a 10-fold excess of $(EA)_2$, in order to simplify the evaluation of the association kinetics. The ratio of bound to total ED was obtained from the analysis of FCS measurements. The normalized autocorrelation curves of free and bound ED are shown in Fig. 1. The faster component with a relaxation time of 2–4 μs is unchanged in FCS measurements of unbound and bound ED. Thus the fast effect is attributed to dynamic processes or the Cy5-dye itself. Such a fast process also appeared with only Cy5 dye and has been interpreted by Jerker Widengren [15] as being *trans–cis* isomeration and triplet formation of the cyanine dye. The mean diffusion time through the focus of approximately 1 fl was 350 μs for free ED and 1 ms for bound ED which corresponds well to a complex of ED and $(EA)_2$, assuming the molecules are spherical in shape. The ratio of bound to total ED increased as an exponential function of time with the characteristic time constant τ and the amplitude A:

$$f(t) = A\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] \quad (2)$$

A typical time course for the association is shown in Fig. 2. Association could be observed at even the lowest ED concentration (1 nM). ED-binding is saturated down to 0.13 μM $(EA)_2$. These results are consistent with those of Zabin [6], who reports a binding constant of 1.2×10^9 M^{-1} for the association of an ED-analog (α-complementing peptide CB2) with the native *E. coli* lacZ-derived $(EA)_2$-analog (M15 β-galactosidase fragment). When binding was saturated, roughly 55% of the available ED molecules were bound to native $(EA)_2$. This means that a considerable percentage of ED is not able to be bound, at least under these experimental conditions. This low maximum degree of association may be caused by ED conformations which are unable to be bound by $(EA)_2$. Association experiments were carried out using 1, 5 and 10 nM ED, and 9.9 μM $(EA)_2$ (Table 1). The time constants for the kinetics did not differ significantly at the three different ED concentrations, which is consistent with a bimolecular association mechanism with the dynamics of the reaction being determined by the species in over-abundance [$(EA)_2$ in this study]. The corresponding time constant was calculated to be 8.8 ± 1.7 min in all cases. Several association experiments carried out on different days proved the reproducibility of the values obtained for the time constants (Table 1). In other experiments, the ED concentration was kept constant at 5 and 10 μM whereas the concentration of $(EA)_2$ was varied from 0.13 to 9.9 μM. The higher the $(EA)_2$ concentration, the faster the association reaction.

Table 1
Time constants of ED-$(EA)_2$ association kinetics with 9.9 μM $(EA)_2$ and 1, 5, 10 nM ED, respectively

[ED]/nM	τ/min
1	8.2
1	7.0
5	8.2
5	12
5	7.9
10	9.9
	8.8 ± 1.7

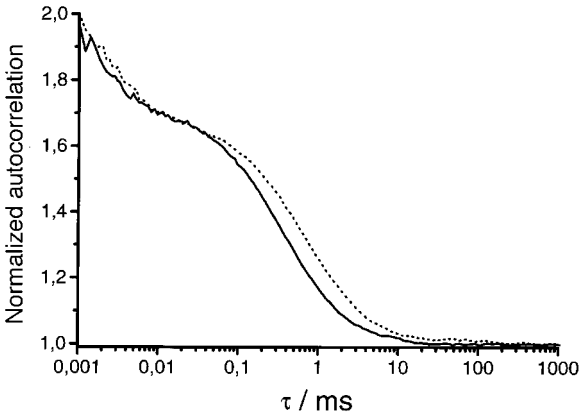

Fig. 1. Normalized autocorrelation curves of free ED (solid line) and ED which is bound to $(EA)_2$ (dotted line, 60% complex).

3.2. Reaction mechanisms

The protein EA has been shown to exist predominantly as a dimer in the concentration range used in this study [16,17]. Therefore all reaction mechanisms discussed here will pertain to the initial binding of the dimer $(EA)_2$ to ED and are summarized in Table 2. The most simple conceivable mechanism which is able to describe the experimental data is a one-step reaction mechanism (model 1).

Since the concentration of $(EA)_2$ was at least 10 times that of ED, the reaction took place under pseudo first order conditions. The time course of the reaction could be described by a mono-exponential function. All experimental association data could be described satisfactorily by model 1 yielding different time constants for different concentrations of $(EA)_2$. The reciprocal of the relaxation time τ for the association kinetics depends on $(EA)_2$ concentration according to the following equation [11]:

$$\frac{1}{\tau} = k_{ass}[(EA)_2]_0 + k_{diss} \quad (3)$$

According to model 1, the slope of the linear regression corresponds to the association rate

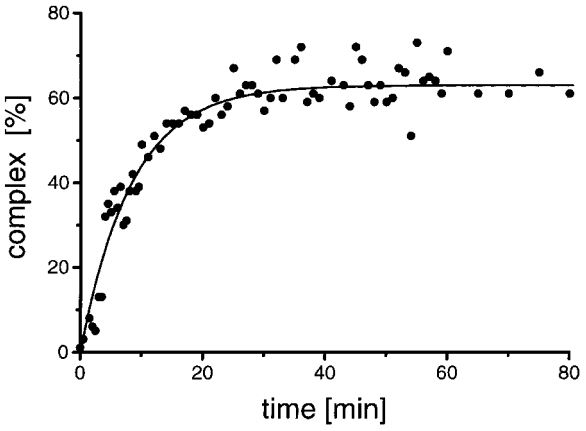

Fig. 2. Association kinetics of 5 nM ED and 9.9 μM $(EA)_2$. The percentage of ED/$(EA)_2$ complex is plotted as a function of time. The kinetics of this reaction can be analyzed from a timed series of FCS experiments. The data were fit to a single exponential with time constant $\tau = 7.9$ min.

constant, k_{ass}, which is determined to be 207 M^{-1} s^{-1} (Fig. 3). The dissociation rate, k_{diss}, cannot be obtained with sufficient accuracy from the intercept of this function because its value is too close to zero (due to the high binding constant [6]) and the standard deviation of the intercept is larger than the dissociation rate. However, it can easily be deduced that k_{diss} is smaller than 2×10^{-4} s^{-1}.

Though model 1 is pleasing in its simplicity, the magnitude of the association rate constant leads us to consider a more realistic reaction mechanism. The association rate constant $k_{ass} = 207$ M^{-1} s^{-1} is judged to be too small for the initial step of the bimolecular association of ED and $(EA)_2$ and, hence, will be defined as the apparent association rate Table 3. A binding constant of 10^9 M^{-1} [6] would suggest a much higher rate of association. For a diffusion-controlled bimolecular association, the rate constant of the diffusion-controlled association reaction is expected to be in the order of 10^7 M^{-1} s^{-1}, according to Smoluchowski [7]. This rate constant is orders of magnitude higher than the apparent association rate deduced from our experiments, which refutes the relevance of model 1.

The data can be explained more reasonably by a reaction mechanism with a fast pre-equilibrium, i.e. with the rate constants k_{1on} and k_{1off} being much larger than k_{2on} and k_{2off} (model 2). ED-$(EA)_2^*$ denotes an intermediate which is slowly converted into the active complex ED-$(EA)_2$. In FCS experiments, it is only the slow dynamics of the second step, described by the rate constant k_2, which are observed, provided there is no change in the specific fluorescence intensity per particle as a result of binding, and if the amount of ED-$(EA)_2^*$ formed during preequilibrium is small. We observed no significant change in fluorescence intensity per particle upon binding. The measured counts per molecule were (26 ± 3) and (28 ± 3) kHz for ED in absence and presence of 9.9 μM $(EA)_2$, respectively; and the presupposition of a low intermediate concentration is met if the reverse rate constant, k_{1off}, is high. The resulting fast pre-equilibrium generates and consumes intermediate molecules very rapidly. In this case, the apparent association rate (k_{app}) is $k_{1on}/k_{1off} \times k_{2on}$. With a diffusion-controlled association rate, k_{1on}, of 10^7 M^{-1} s^{-1} and a fast reverse reaction with a k_{1off} of 10^6 s^{-1}, a rate limiting secondary step with a k_{2on} of 20.7 s^{-1} could be calculated using the apparent association rate ($k_{app} = 207$ M^{-1} s^{-1}). Since a saturation of ED binding is achieved even at the lowest $(EA)_2$ concentrations used, the k_{2off} is estimated to be below 5×10^{-6} s^{-1} based on simulations of the time course of complex formation according to model 2. From a simulation with these time constants the intermediate complex concentration is calculated to be approximately 0.1% of total complex concentration after 30 s, where the first data point was measured using FCS, and vanishes during the course of the reaction. The experimental and simulated inverse time constants for these rate constants are shown in Fig. 3. Thus, the experimental data pertaining to the association kinetics can be explained by an association reaction in which a fast pre-equilibrium is followed by a much slower secondary step which is characterized by a low number of transfers per second and is rate limiting.

Fowler and Zabin [8] have shown that the active form of β-galactosidase is a tetramer. The wild-type tetramer is shown to be stabilized by a

Table 2
Proposed models of ED/$(EA)_2$ reaction mechanisms with corresponding systems of ordinary differential equations (ODEs)

Model	Chemical equation	System
1	$ED + (EA)_2 \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} ED-(EA)_2$	$\frac{d[ED-(EA)_2]}{dt} = k_{on}[ED][(EA)_2] - k_{off}[ED-(EA)_2]$ $\frac{d[ED]}{dt} = \frac{d[(EA)_2]}{dt} = -\frac{d[ED-(EA)_2]}{dt}$
2	$ED + (EA)_2 \underset{k_{1off}}{\overset{k_{1on}}{\Leftrightarrow}} ED-(EA)_2^* \underset{k_{2off}}{\overset{k_{2on}}{\Leftrightarrow}} ED-(EA)_2$	$\frac{d[ED-(EA)_2]}{dt} = k_{2on}[ED-(EA)_2^*] - k_{2off}[ED-(EA)_2]$ $\frac{d[ED-(EA)_2^*]}{dt} = -\frac{d[ED-(EA)_2]}{dt} + k_{1on}[ED][(EA)_2] - k_{1off}[ED-(EA)_2^*]$ $\frac{d[ED]}{dt} = \frac{d[(EA)_2]}{dt} = -\frac{d[ED-(EA)_2^*]}{dt} - \frac{d[ED-(EA)_2]}{dt}$
3	$ED + (EA)_2 \underset{k_{1off}}{\overset{k_{1on}}{\Leftrightarrow}} ED-(EA)_2 \overset{+ED\text{-}(EA)_2}{\underset{k_{2off}}{\overset{k_{2on}}{\Leftrightarrow}}} \{ED-(EA)_2\}_2$	$\frac{d[\{ED-(EA)_2\}_2]}{dt} = k_{2on}[ED-(EA)_2]^2 - k_{2off}[\{ED-(EA)_2\}_2]$ $\frac{d[ED-(EA)_2]}{dt} = k_{2off}[\{ED-(EA)_2\}_2] - 2k_{2on}[ED-(EA)_2]^2 + k_{1on}[ED][EA)_2] - k_{1off}[ED-(EA)_2]$ $\frac{d[ED]}{dt} = \frac{d[(EA)_2]}{dt} = -k_{1on}[ED][(EA)_2] + k_{1off}[ED-(EA)_2]$
4	$ED + (EA)_2 \underset{k_{1off}}{\overset{k_{1on}}{\Leftrightarrow}} ED-(EA)_2 \overset{+(EA)_2}{\underset{k_{2off}}{\overset{k_{2on}}{\Leftrightarrow}}} ED-(EA)_4$	$\frac{d[ED-(EA)_4]}{dt} = k_{2on}[ED-(EA)_2][(EA)_2] - k_{2off}[ED-(EA)_4]$ $\frac{d[ED-(EA)_2]}{dt} = -\frac{d[ED-(EA)_4]}{dt} + k_{1on}[ED][(EA)_2] - k_{1off}[ED-(EA)_2]$ $\frac{d[ED]}{dt} = -\frac{d[ED-(EA)_2]}{dt} - \frac{d[ED-(EA)_4]}{dt}$ $\frac{d[(EA)_2]}{dt} = \frac{d[ED]}{dt} - \frac{d[ED-(EA)_4]}{dt}$
5	$ED + (EA)_2 \underset{k_{1off}}{\overset{k_{1on}}{\Leftrightarrow}} ED-(EA)_2^* \underset{k_{2off}}{\overset{k_{2on}}{\Leftrightarrow}} ED-(EA)_2 \overset{+(EA)_2}{\underset{k_3}{\rightarrow}} ED\text{–}(EA)_4$	$\frac{d[ED-(EA)_4]}{dt} = k_3[ED-(EA)_2][(EA)_2]$ $\frac{d[ED-(EA)_2]}{dt} = k_{2on}[ED-(EA)_2^*] - k_{2off}[ED-(EA)_2] - \frac{d[ED-(EA)_4]}{dt}$ $\frac{d[ED-(EA)_2^*]}{dt} = k_{2off}[ED-(EA)_2] + k_{1on}[ED][(EA)_2] - (k_{1off} + k_{2on})[ED-(EA)_2^*]$ $\frac{d[ED]}{dt} = -k_{1on}[ED][(EA)_2] + k_{1off}[ED-(EA)_2^*]$ $\frac{d[(EA)_2]}{dt} = \frac{d[ED]}{dt} - \frac{d[ED-(EA)_4]}{dt}$

Table 3
Apparent association rates k_{ass} for denatured and native $(EA)_2$ (both 9.9 μM) and ED

EA variant	Apparent association rate (M^{-1} s^{-1})
Denatured	140 ± 27
Native	207 ± 13

four-helix bundle which is formed at the activation interface [9]. Such a tetramer could be formed by reaction mechanisms shown in models 3–5.

Model 5 is an extension of model 2. It reduces to model 2 if k_3 is very small. This mechanism is characterized by the formation of a tetramer which is bound to one ED molecule. The fact that only one ED molecule is contained in complexes with $(EA)_2$ is proven by FCS measurements showing no change in the concentration of fluorescent molecules during the association reaction. This result agrees with results of Gallagher [2] who found that two ED analoges bound to both the dimer and the tetramer of EA in a ratio of 1:1.

Since a difference of a factor of two in molecular weight would correspond to only a 20% change in the corresponding translational diffusion time (assuming the particles have a spherical shape) and the relative error of the measurement of the diffusion time of the complex is approximately 10%, which is in the range of the expected difference between the diffusion time of dimer and tetramer, and the conformation of the molecules could even deviate from spherical shape, the tetramer and the dimer can hardly be distinguished using FCS, which measures translational diffusion times through the observation volume. Under these conditions, the sum of complexes would be measured by FCS approximately as a single slow component. This assumption has been supported by theoretical examination of ternary mixtures consisting of 40% unbound ED, and varying relative concentration fractions of ED-$(EA)_2$ and ED-$(EA)_4$. FCS curves of these mixtures were simulated according to Eq. (1) (with $T = 0.2$, $\tau_T = 4$ μs, $\tau_{ED} = 390$ μs, $\tau_{ED-(EA)2} = 1.0$ ms, $\tau_{ED-(EA)4} = 1.2$ ms) and fit to a two-component model considering the two ED-complexes as a single component. The deviation of calculated binding degrees of ED is less than 14%.

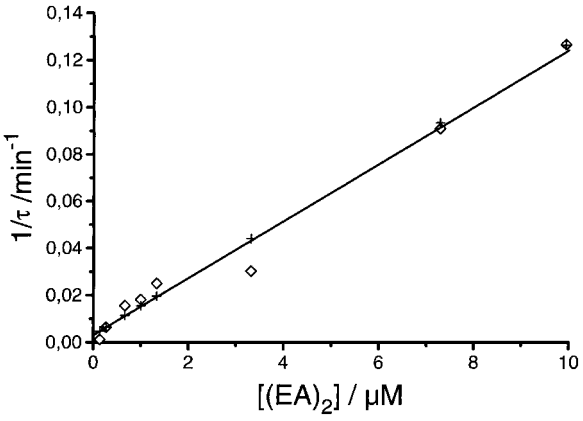

Fig. 3. Kinetics of ED/$(EA)_2$ binding. The inverse of the reaction time constant τ is plotted as a function of $(EA)_2$ concentration. The $(EA)_2$ concentration varied from 0.13 to 9.9 μM. The ED concentration was kept constant at 5 nM. The inverse of the reaction time constant shows a linear dependence on $(EA)_2$ concentration. From the slope of this graph an apparent association rate, k_{ass}, of 207 M^{-1} s^{-1} could be calculated. Open diamonds denote experimental and crosses calculated data for model 2 employing $k_{1on} = 10^7$ M^{-1} s^{-1}, $k_{1off} = 10^6$ s^{-1}, $k_{2on} = 20.7$ s^{-1} and $k_{2off} = 5 \times 10^{-6}$ s^{-1}.

Therefore assuming that the initial bimolecular association step is a fast pre-equilibrium which leads to the formation of the intermediate ED-$(EA)_2^*$, and if this intermediate is slowly interconverted into the complex ED-$(EA)_2$ by conformation and change with a small reverse rate, k_{2off}, then the sum of dimeric and tetrameric complexes is independent of k_3, and model 2 and model 5 cannot be distinguished using FCS. The experimental data are, therefore, also consistent with tetramer formation (model 5). However, by FCS, it is not possible to conclusively prove the generation of tetramers as postulated by others.

Models 3 and 4, which appear to be reliable reaction mechanisms for the tetramer formation of EA at first glance, cannot explain the data and are discussed in the following section:

The second step in model 3 is the dimerization of an ED-$(EA)_2$ dimer. Hence, the concentration of fluorescent molecules should decrease by a factor of two. FCS measurements, which precisely determine the number of fluorescent molecules per observation volume and thus the concentra-

tion, show that a more slowly diffusing fluorescent complex is formed, but the concentration of the fluorescent molecule does not change. Hence, model 3 can be excluded as a realistic reaction mechanism.

Model 4 is a two-step reaction mechanism with formation of ED-$(EA)_2$ in the first step. The second step of the model is characterized by the binding of $(EA)_2$ to ED-$(EA)_2$ yielding a tetramer with regard to EA which has bound one ED. Model 4 simplifies to model 1 if k_{2on} is small, meaning that the second step becomes a negligible reaction pathway. Model 1 and model 4 cannot be distinguished, despite the additional tetramer species in model 4, if k_{1off} is very small (e.g. 10^{-5} s^{-1}), meaning that the first step is practically irreversible, thereby preventing substantial dissociation of dimeric complex. This means that the sum of dimeric and tetrameric complexes which is measured as one fluorescent species using FCS is independent of k_{2on} and k_{2off}. Since the measured k_{app} is as low as 207 M^{-1} s^{-1}, this case is not relevant. If significant amounts of tetramer are assumed to be formed, a large k_{2on} is to be expected. Furthermore, if the rate constant k_{1on} is diffusion-dependent, k_{1off} must be rather large in order to obtain kinetics which are as slow as the experimental time course of α-complementation. Therefore the kinetics of model 4 were simulated with rate constants $k_{1on} = 10^7$ M^{-1} s^{-1}, $k_{1off} = 10^6$ s^{-1}, $k_{2on} = 5 \times 10^6$ M^{-1} s^{-1} and $k_{2off} = 10^{-5}$ s^{-1}. The simulated kinetics were in the range of the experimental data (Fig. 4). A plot of the inverse of τ vs. $(EA)_2$ concentration showed a non-linear increase and thus deviates qualitatively from the experimental data. In particular, if the chemical equilibrium is shifted to favor tetramer formation, the tetramer concentration will be greater than that of dimeric complexes. Then FCS measurements indicate the increase in tetramer concentration: Tetramer formation is dependent both on the association of $(EA)_2$ with ED, and of $(EA)_2$ with ED-$(EA)_2$. Therefore an increase in $(EA)_2$ concentration accelerates both reaction steps resulting in a more than proportional increase in the kinetics of tetramer and thus total complex formation. The discrepancy between simulated and experimental

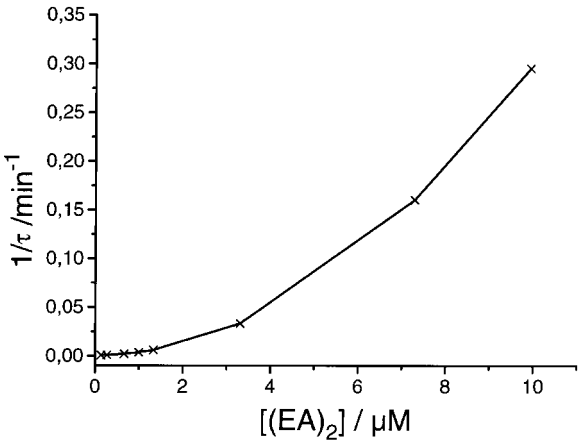

Fig. 4. Simulated relaxation times τ according to model 4 using the following parameters: $k_{1on} = 10^7$ M^{-1} s^{-1}, $k_{1off} = 10^6$ s^{-1}, $k_{2on} = 5 \times 10^6$ M^{-1} s^{-1} and $k_{2off} = 10^{-5}$ s^{-1}.

data leads to the rejection of model 4 if the first association step is very rapid, or is diffusion-controlled, and if a significant amount of tetramer is formed.

Since models 1, 3, and 4 can be excluded by arguments that they result in rates of association which are far lower than those expected for diffusion-controlled association, no change in particle number is observed, and discrepancies in the dependence of τ on the concentration between theoretical and experimental data, models 2 and 5 turn out to be the most reasonable. Both models imply a fast pre-equilibrium followed by a slow formation of the final complex which is enzymatically active. Since there is strong evidence that the active enzyme is the tetramer [8,9], we propose model 5 as being the most realistic reaction model. This model comprises a fast pre-equilibrium, two dimeric intermediates and an irreversible transformation into the tetramer which contains only one ED.

3.3. Comparison of native $(EA)_2$ and denatured $(EA)_2$

It was previously known that denatured $(EA)_2$, in contrast to the native form, has no detectable enzyme activity. Until now, it was unclear whether this was due to altered binding characteristics or

a direct alteration of enzymatic activity. Using FCS it is possible to study the association reaction independent of activity measurements.

The mean degree of binding — calculated from FCS-experiments — for denatured $(EA)_2$ was 46% and that for native $(EA)_2$ 55%. The time constants of the association kinetics of denatured $(EA)_2$ were 1.5 times greater than those observed with native $(EA)_2$. The deduced apparent association rate for experiments with native $(EA)_2$ was 207 M^{-1} s^{-1}, and the apparent association constant for denatured $(EA)_2$ was approximately 140 M^{-1} s^{-1}. Though enzymatic activity was completely lost, the association kinetics could still be measured using FCS and changed to only a small extent.

Assays which utilize the activity of β-galactosidase after reconstitution, e.g. CEDIA^{TM}, are dependent on the stability of the components ED and $(EA)_2$. Hence, costly quality controls must be carried out in order to ensure adequate performance of such an assay. Since the lifetime of enzymatic activity is limited, such an assay may have a shelf life of only a few weeks. Our results show that the binding of ED and $(EA)_2$ is affected to only a slight degree by the loss of enzymatic activity. In fact, even artificially denatured $(EA)_2$ can be used for an assay which yields reproducible kinetic data. Thus, this type of assay can be evaluated using FCS without concern for the viability of enzymatic activity. The advantage of a robust assay is thus combined with intrinsic advantages of the FCS method, namely small sample volumes of 1–20 μl (detection volume 10^{-15} l), high sensitivity (down to 20 pM), and, therefore, the requirement of as little as 2×10^{-17} mol of fluorescent ED per assay.

4. Conclusions

FCS enables a scientist to measure kinetics under native conditions in the nanomolar and subnanomolar range. It has been shown that FCS is a rapid and reliable method for the analysis of kinetic reactions.

The formation of the β-galactosidase tetramers has been measured for both the denatured and the active form of $(EA)_2$. The binding of the two reaction partners, ED and $(EA)_2$, is the first step of this reaction cascade. The association reaction could be easily followed and comparable degrees of binding were measured in all experiments. The experimental data led to the conclusion that the kinetics of the reaction are best described by a fast preequilibrium state and a subsequent slower formation of the dimeric complex. Simulations have shown that a subsequent bimolecular association of ED-$(EA)_2$ and excess $(EA)_2$ yielding active tetramers is also consistent with the experimental data. Since it has been found that the active β-galactosidase enzyme exists as a tetramer, we conclude that the second slow reaction step involves tetramerization (model 5).

Comparison of native and denatured $(EA)_2$ revealed similar association kinetics for both forms and, therefore, probably the same reaction mechanism. It can be concluded that denaturation of the protein does not affect the binding domain significantly, rather the catalytic site.

The binding of two or more molecules to one another is a property universally used to ascertain information about biological events. FCS provides direct access to binding events in real time — and also provides the means to immediately determine an array of pertinent biochemical information. CEDIA^{TM} type assays employ the enzyme reaction of reconstituted β-galactosidase. Such an assay would be susceptible to a loss of sensitivity due to the half-life of enzymatic activity compared to an FCS-based assay which directly measures binding rather than enzymatic activity. Since FCS provides direct insight, rather than indirect measurements of biochemical interactions, it promises to become a powerful tool in future biochemical and cell-based assay system development.

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