PULSE FLUORIMETRIC STUDY OF LABELLED ACTIN–DNase I COMPLEX

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1. Introduction

Physicochemical investigations of muscle actin conjugated with fluorescent probe have been performed extensively [1–3]. Meanwhile the discovery of the complex formation of actin with DNase I has given a clue to the study of actin from its function in cell activity [4]. The fluorescence technique has the advantage of determining the binding constant even at very dilute protein concentration. Muscle actin was labelled with 1,5-IAEDANS and the anisotropy decay of AEDANS-G–DNase was measured by single photoelectron counting method. The rotational correlation time obtained was a function of molecular volume which increased with complex formation. Then from fluorescence correlation time determined as a function of the G-actin : DNase I ratio, the binding constant (K_b) 1.46 × 10^6 M^{-1}, was obtained.

2. Materials and methods

Extraction of rabbit skeletal muscle actin and its labelling with 1,5-IAEDANS was performed as in [5]. Actin concentration was determined by A_{280} = 1.11 $(mg/ml)^{-1} . cm^{-1}$ and corrected for the absorption of 1,5-IAEDANS estimating $\epsilon_{280} = 0.25\ \epsilon_{336}$. For molecular weight of G-actin, 42 300 was used. DNase I was purchased from Worthington Corp. (code D) and purified with Sephacryl S-200 (Pharmacia) in the presence of 0.1 mM PMSF. The concentration of DNase I was determined using both A_{280} = 1.23 $(mg/ml)^{-1} . cm^{-1}$ and mol. wt 31 000. Labelling ratio, 33.5% was obtained using A_{336} = 6100 $M^{-1} . cm^{-1}$ [6]. 1,5-IAEDANS was purchased from Aldrich-USA and ATP from Sigma. The pulse fluorimeter was that in [7]. The excitation wavelength, 357 nm, was selected by MTO-357 interference filter and emission wavelength, 450 nm, was selected by SC-41 cut-off filter and BPB-45 bandpath filter (Fuji Colour Film). The temperature was controlled at 2.5°C. The components of transient polarized fluorescence $i_{\parallel}(t)$ and $i_{\perp}(t)$ give the following functions:

$$s^{ex}(t) = i_{\parallel}(t) + 2\, i_{\perp}(t)/\beta$$

$$d^{ex}(t) = i_{\parallel}(t) - i_{\perp}(t)/\beta \qquad (1)$$

$$r^{ex}(t) = d^{ex}(t)/s^{ex}(t)$$

The value of β was obtained by the methods shown in [7]. The fluorescence decay of AEDANS-G and AEDANS-G–DNase was analysed as a sum of two exponential functions [5]. According to [8], the response function was determined with a reference compound 1,1,4,4-tetraphenyl-butadiene of which fluorescence decay time was put to 1.78 ns at 10°C in cyclohexane [9]. Parameters of the anisotropy decay were calculated as in [5]. The interaction of actin with DNase I was analysed by the theory of multiple equilibria in which it is assumed that DNase I,D, has several binding sites of actin A:

$$DA_i + A \rightleftarrows DA_{i+1}\ (i=0,1,2,\ldots,n) \qquad (2)$$

The av. no. (ν) actin molecules bound/mol DNase I is given as follows:

Abbreviations: 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; PMSF, phenylmethane-sulfonyl-fluoride; DNase I, deoxyribonuclease I; AEDANS-G, 1,5-IAEDANS-labelled G-actin; AEDANS-G–DNase, complex of AEDANS-G and DNase I

$$\nu = (\sum_{i=0}^{n} i\,[DA_i])/(\sum_{i=0}^{n} [DA_i]) =$$

$$(C_\circ - C)/D_\circ = (1 - x_G)C_\circ/D_\circ \qquad (3)$$

where C is the molar concentration of free AEDANS-G, $C_\circ$ is the total molar concentration of AEDANS-G, x_G is the mole fraction of free AEDANS-G; $C = x_G C_\circ$ and $D_\circ$ is the total molar concentration of DNase I. The apparent binding constant K is given by the following equation:

$$K = \frac{\nu}{C(\nu_{max} - \nu)} \qquad (4)$$

where ν_{max} is the maximum number of binding sites of DNase I.

3. Results

We found that the decay constants of fluorescence from AEDANS-G were the same as those in [5] and were kept almost constant even after the addition of DNase I (table 1). Therefore, it is reasonable to consider that the chromophore fluorescence is identical in both AEDANS-G and AEDANS-G–DNase. The anisotropy decay of AEDANS-G could be approximated with a single exponential term giving the fundamental anisotropy 0.286 and the correlation time 45.5 ns, while in [5] another term of very short correlation time was included. Then the anisotropy decay of AEDANS-G–DNase can be written as follows:

$$R(t) = x_G r_G^0 \exp(-t/\theta_G) + (1 - x_G) r_G^0 \exp(-t/\theta_C) \qquad (5)$$

where r^0 is the fundamental anisotropy, θ is the rotational correlation time, and subscript G and C refer to the free and complex states of AEDANS-G. Fixing r_G^0 at 0.286 and θ_G at 45.5 ns, the anisotropy decays of AEDANS-G–DNase at several ratios of AEDANS-G to DNase I were analysed with eq. (5). We found that neither r_C^0 nor θ_C showed any significant dependence on the ratio: Their mean values were $r_C^0 = 0.282$ and

Table 1

DNase I (mg/ml)	a_1	τ_1	a_2	τ_2	χ_S^2
0	0.133	10.9	0.775	25.1	9.86
0.073	0.150	9.32	0.787	24.9	1.32
0.14	0.141	10.3	0.762	25.5	1.28
0.27	0.194	8.34	0.766	25.3	1.30
0.41	0.175	9.30	0.772	25.0	1.39
0.60	0.181	6.20	0.792	25.0	2.02

Parameters of fluorescence decay of AEDANS-G and AEDANS-G–DNase at 2.5°C in 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 1 mM NaN_3 and 2 mM Tris-acetate (pH 7.0); concentration of AEDANS-G was 0.3 mg/ml:

$$S(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$$

$$s^c(t) = g(t) * S(t)$$

where $g(t)$ is the response function and parameters were determined with convolution of $S(t)$ by the least squares method. χ_S^2 is the weighted mean square of the difference between $s^{ex}(t)$ and $s^c(t)$

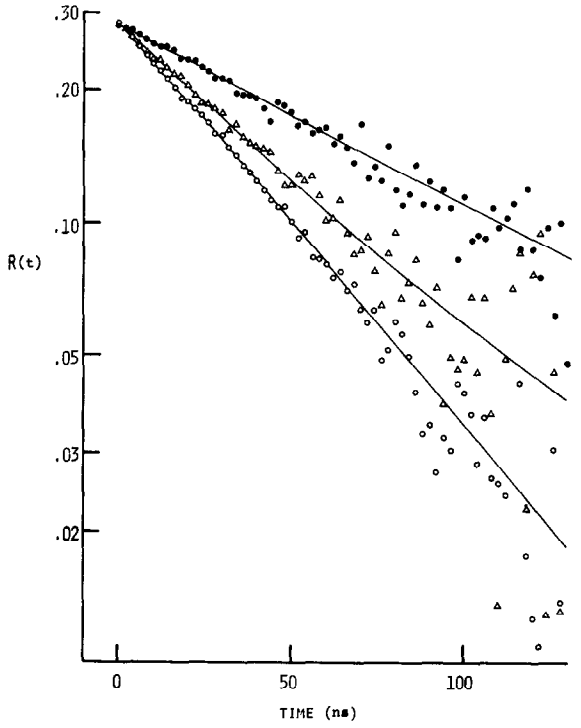

Fig.1. Anisotropy decay of AEDANS-G and AEDANS-G–DNase (at 25°C). Time equal zero was taken as the peak of lamp intensity. The solid lines represent the calculated anisotropy and the symbols are for experimental data at following conditions: (○) in the absence of DNase I; (△) 0.073 mg DNase I/ml; (●) 0.43 mg DNase I/ml; AEDANS-G was fixed at 0.30 mg/ml. Plots are shown at every two channels. The solvent condition is described in table 1.

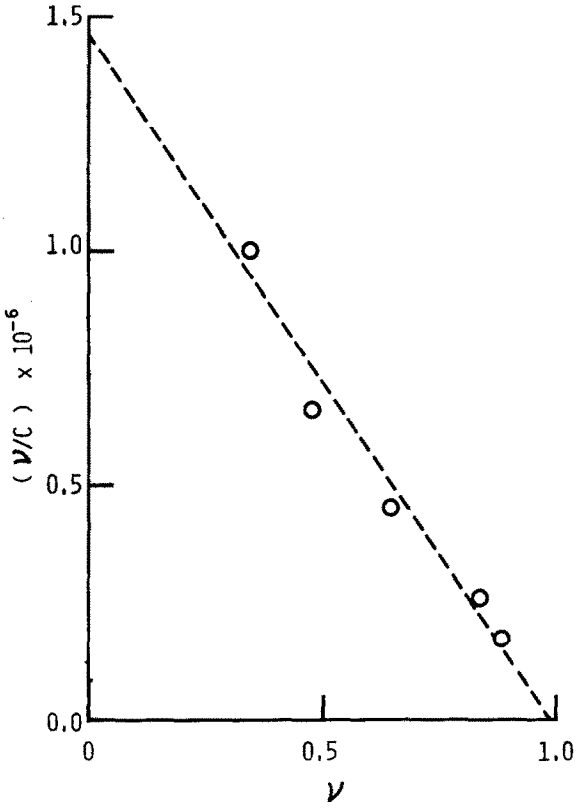

Fig.2. Plots of ν/C versus ν.

θ_C = 113.8 ns. Then putting r_G^0 = 0.286, r_C^0 = 0.282, θ_G = 45.5 ns and θ_C = 113.8 ns, x_G was evaluated again for each AEDANS-G–DNase solution.

With the x_G values obtained, the binding constant was estimated with eq. (4). As shown in fig.2, plots of ν/C against ν gave a straight line. The values of ν_{max} and K were determined from the linear regression and we obtained ν_{max} = 0.98 and $K = 1.46 \times 10^6$ M^{-1}.

4. Discussion

The fluorescence decay parameters of AEDANS-G obtained in the present study are in agreement with the results in [5], but difference from the results in [10] is obvious. It may be explained with difference in the labelling condition.

In agreement with [11], the analysis of fig.2 shows 1:1 association of AEDANS-G and DNase I. The fluorescence decay time and the fundamental anisotropy of AEDANS-G were not affected with the association of DNase I. Therefore the binding constant of AEDANS-G and DNase I obtained in the present study is probably very close to that of G-actin and DNase I, although the labelling may affect the binding to some extent. The binding constant 5×10^8 M^{-1} of G-actin and DNase I was given in [12], but their method of determination is not clear. Further studies of this kind are required to resolve this problem.

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