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Flexibility of the aldolase molecule measured using quenching-induced variations of the Forster distance for fluorescence energy transfer

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Abstract. The range of flexibility of the rabbit muscle aldolase molecule was studied using fluorescent labelled aldolase. The protein molecule was specifically labelled on the opposite sites of the enzyme subunit with the fluorescence energy donor and acceptor residues. Labelled aldolase with full enzymatic activity was used as a tool in the FRET studies between IAEDANS – donor or Cys-289 and IAF – acceptor on Cys-239. A range of Forster distance (R) was obtained by collisional quenching of the donor emission. The experiments of donor fluorescence quenching with wide range of acrylamide concentrations have shown the changes of donor–acceptor distances. In the absence of quencher the D–A distance distribution in characterized by an average value of 40·4 Å, and a half-width of 0·13 Å. A dramatic increase in half-width to 17·7 Å is observed after expositions of the enzyme to high acrylamide concentrations (0·13 M–0·68 M).

Keywords. Aldolase; protein dynamics; Forster distance; fluorescence quenching.

1. Introduction

Recently there is growing interest in labelling of protein molecules with fluorescence donor and acceptor probes such labelled proteins has been widely used as tools in FRET studies in the distance measurements between specific sites on biological macromolecules. However, there exist many situations where a single D to A distance is not expected for a given sample of molecule such as in the case of denatured protein. The steady-state transfer efficiency measured with a single D–A pair cannot reveal the D–A distance distribution. Information on the distributions can be obtained from fluorescence lifetime measurements with laser sources to provide good resolution of short and/or complex decays. In this report collisional quenching was used to decrease the quantum yield of the donor which in turn decreases the value of R_0 as described by Gryczyński *et al* (1980) and Lakowicz *et al* (1988). A range of R_0 values yields good estimates of the D–A distance distributions. Recently, we described the preparation of a derivative of rabbit muscle aldolase A specifically labelled with the

Abbreviations: IAEDANS, 5-(2-((iodoacetyl)amino)ethyl)aminonaphthalene-1-sulphonic acid; IAF, 5-iodoacetamidofluorescein; aldolase-AEDANS, aldolase labelled with IAEDANS at Cys-289; aldolase-AEDANS-IAF, aldolase labelled with AEDANS at Cys-289 and IAF at Cys-239; FRET, fluorescence energy transfer.

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fluorescent donor at Cys-289 and acceptor at Cys-239. Based upon energy transfer experiments the distance between these two probes in the native aldolase molecule in about 43 Å which is comparable to aldolase subunit diameter (Dobryszycki *et al* 1989). In this report labelled aldolase was used to study the effect of acrylamide on the protein structure. Eftink and Ghiron (1987) studies revealed no significant acrylamide-protein interactions and did not support the claim of Blatt *et al* (1986) that acrylamide may bind to certain proteins. It is shown from our results that at about 0·2 M acrylamide concentration the aldolase molecule becomes much more flexible although the average distance ($\bar{r}$) between donor and acceptor is maintained.

2. Materials and methods

2.1 *Chemicals*

Aldolase with a specific activity of 14–15 units/mg was prepared according to Penhoet *et al* (1969). The aldolase activity was assayed spectrophotometrically at 25°C in 100 mM Tris, 1 mM EDTA (pH 7·5) using a coupled enzyme assay (Blostein and Rutter 1963). The enzyme concentration was calculated assuming an $A^{1\%}_{1cm}$ value of 9·1 (Baranowski and Niederland 1949). IAF and IAEDANS were purchased from Molecular Probes, Inc. (Eugen, USA). All other chemicals used were of the best quality available. The outline of the aldolase labelling procedure has been previously presented (Dobryszycki *et al* 1989) and the details are as described earlier (Dobryszcki *et al* 1990). The probes were incorporated into aldolase with the following stoichiometry of labelling: IAEDANS, 0·68–0·73; IAF, 0·15–0·38 per aldolase subunit.

2.2 *Theory*

The quencher-dependent Forster distances (R_0^Q) are calculated from the decrease in quantum yield (Φ_d^Q) due to the collisional component, taken from the Stern–Volmer plots of the quenching given by:

$$\Phi_d^Q = \frac{\Phi_d^0}{1 + K_D[Q]} \tag{1}$$

where K_D is the dynamic quenching constant, $[Q]$ is the quencher concentration, and Φ_d^0 is donor quantum yield in the absence of quencher. The quenched quantum yields are used to calculate the Forster distance.

$$R_0^Q = R_0(\Phi_d^Q/\Phi_d^0)^{1/6}, \tag{2}$$

where R_0 is the Forster distance calculated according to Forster (1949). We assumed the D–A distributions to be described by a Gaussian:

$$P(r) = \frac{r^n}{\sigma(2\pi)^{\frac{1}{2}}} \exp\left[-\frac{1}{2}\left(\frac{r-\bar{r}}{\sigma}\right)^2 \right] \tag{3}$$

where r is the D–A distance, $\bar{r}$ is the average, and σ the standard deviation of the distribution, and $n = 0,1,2$. Full-width at half-maximum, hw = $2{,}354\sigma$. The parameters

describing the D–A distribution were determined by nonlinear least squares. For any values of the assumed parameters ($\bar{r}$ and σ) the energy transfer efficiency can be predicted using:

$$E_c^Q = \int_{r=r_{min}}^{\infty} \frac{P(r)\cdot(R_0^Q)^6}{(R_0^Q)^6 + r^6}\,dr. \tag{4}$$

Fluorescence decays were fit to a single or double exponential decay:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}, \tag{5}$$

where α_i are the preexponential factors and τ_i the decay times. Additionally the calculated time dependence of the fluorescence intensity of the donor in aldolase-AEDANS-IAF was analysed according to Haas *et al* (1988).

$$I(t) = m\int_0^{\infty} P(r)\exp\{(-t/\tau_d)[1 + (R_0/r)^6]\}\,dr, \tag{6}$$

where m is a proportionality constant depending on the system and τ_d is the fluorescence lifetime of the donor in the absence of an acceptor.

2.3 *Methods*

The absorption spectra were measured with an ACTA M VI spectrophotometer (Beckman) at 25°C and Specord M-42 (Jena). The fluorescence steady-state measurements were made with a Perkin–Elmer MPF-44 fluorimeter equipped with corrected spectrum accessories in a 3 mm optical path microcuvette at 25°C. The lifetime measurements were made with an impulse fluorimeter LIF 200 (GDR) with laser source IGT 50 (GDR) ($N_2, \lambda_{EX} = 337$ nm). The parameters describing the assumed distribution are varied to yield the minimum value of χ_R^2:

$$\chi^2 = \frac{1}{v}\sum_Q \left(\frac{E^Q - E_c^Q}{\Delta E}\right)^2, \tag{7}$$

where E^Q are the measured efficiencies at each R_0^Q value, and v is the number of degrees of freedom.

3. Results and discussion

Emission spectra of labelled enzyme (aldolase-AEDANS and aldolase-AEDANS-IAF) are shown in figure 1. In doubly labelled aldolase excited at 345 nm AEDANS fluorescence decreases and amplification of IAF fluorescence is observed, indicating clearly the energy transfer. The effect of acrylamide on FRET is shown in figure 2. The dynamic quenching constant (K_D) calculated from the initial slope of Stern–Volmer plot equals 2·55 M^{-1}. Least squares fits of the fluorescence lifetimes of the donor (τ_d) in the presence of acrylamide (table 1) change from the single exponent to double exponent mode at about 0·2 M acrylamide concentration, so in further investigations

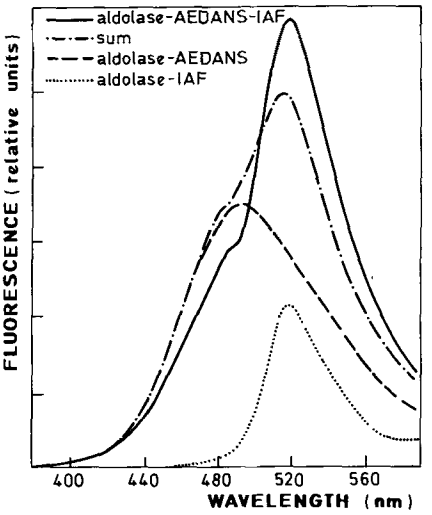

Figure 1. The comparison of the fluorescence emission spectrum of aldolase-AEDANS-IAF (0·68 AEDANS/aldolase subunit, 0·28 IAF/aldolase subunit) and the spectra of aldolase-AEDANS, aldolase-IAF and the sum of both singly labelled derivatives.

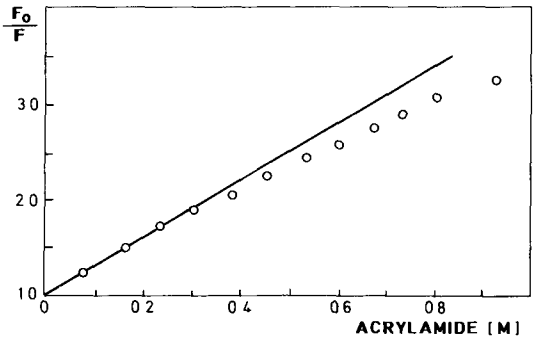

Figure 2. Stern–Volmer plot for acrylamide quenching of aldolase-AEDANS ($c = 1\ \mu M$, 0·45 AEDANS/aldolase subunit, buffer 10 mM Tris, 1 mM EDTA, pH 7·5, temperature 20°C.)

the experimental data were separated into two sets depending on acrylamide concentrations (0–0·29 M and 0·19–0·68 M). As a control, similar experimental data were obtained for aldolase-AEDANS denatured in 8 M urea. The examples of the fluorescence decays of the donor in the aldolase samples in the presence and absence of quencher and in the presence or absence of denaturant are shown in figure 3. The values of the transfer efficiencies for native and denatured aldolase derivatives are well fit by distributions with half-widths of 0·13 Å and 55 Å respectively (figure 3 inset). The very narrow distance distributions for aldolase in the presence of acrylamide (0–0·29 M) (figure 4) suggest that: (a) this concentration of acrylamide does not effect the protein conformation; (b) labelling of the enzyme is very specific. The last conclusion is in good agreement with our previous data based upon chemical modification studies (Dobryszycki *et al* 1988). In the presence of higher acrylamide concentrations (0·13–0·68 M) the broad distance distribution is observed (figure 4b), although the average distance distribution is almost the same. In the case of denatured

Table 1. Fluorescence lifetimes of aldolase-AEDANS in the presence of acrylamide.

Quencher conc. [M]	τ[ns]	Error	τ_1[ns]	α_1	τ_2[ns]	α_2	Error
0·0000	23·87	0·0143	24·51	0·99	24·51	0·01	0·0178
0·0528	17·13	0·0139	17·13	0·98	17·15	0·02	0·0148
0·1049	15·64	0·0105	15·66	0·37	15·63	0·63	0·0111
0·2314	12·44	0·0071	16·34	0·50	9·25	0·50	0·0044
0·3532	11·00	0·0156	13·19	0·79	3·43	0·21	0·0046
0·4704	10·38	0·0201	13·25	0·73	3·13	0·27	0·0051
0·6922	9·54	0·0261	14·19	0·59	3·55	0·41	0·0043
0·8987	9·09	0·0271	13·30	0·60	2·79	0·40	0·0048

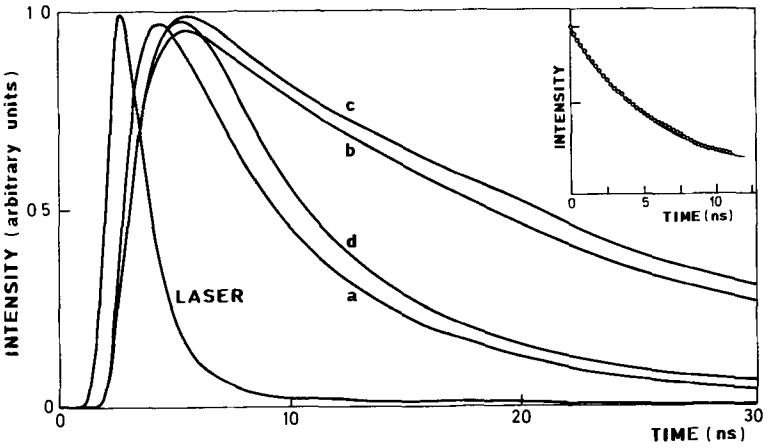

Figure 3. Fluorescence decay of the donor, AEDANS, in aldolase-AEDANS-IAF (**a**) aldolase-AEDANS-IAF (0·73 AEDANS, 0·38 IAF per aldolase subunit), (**b**) aldolase-AEDANS, (**c**) aldolase-AEDANS in 8 M urea and (**d**) aldolase-AEDANS in 0·5 M acrylamide. The experimental curve (**a**) was fitted to a monoexponential decay function (example in inset).

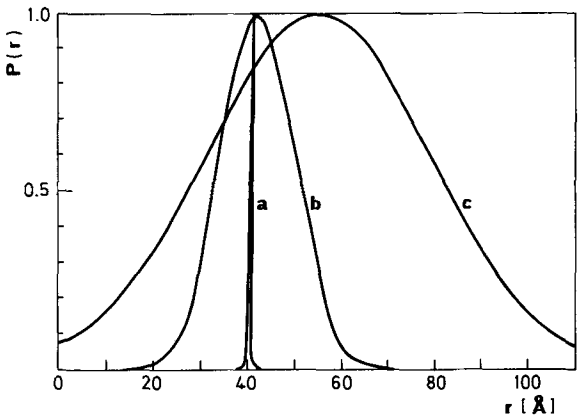

Figure 4. Comparison of the distance distribution for aldolase-AEDANS-IAF ($c = 1$ M, 0·75 AEDANS 0·38 IAF) in acrylamide: (**a**) 0 to 0·29 M, (**b**) 0·13 to 0·68 M and (**c**) in 8 M urea.

aldolase the average distance switches from 40 to 54 Å. The comparison of distance distribution of aldolase-AEDANS-IAF in higher concentration of acrylamide with the data obtained for denatured protein indicates that the initial effect of quencher on aldolase molecule is associated with increased freedom of protein skeleton movement and this, in consequence, may lead to the partial denaturation of protein, particularly during long term exposure to higher acrylamide concentration, because only a small change in D–A distance is observed for aldolase incubated at low concentrations of acrylamide. At present we do not know if the observed increase of aldolase dynamics due to acrylamide is the general phenomenon which can be extended to other proteins. Anyway, our data demonstrate that acrylamide interacts with aldolase which is in discord with Eftink and Ghirons (1987) report. The above method can be successfully used for studying protein dynamics and the effect of external ligands on stability of the native structure of the protein molecule. Further investigations on aldolase dynamics are in progress.

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