FLUORESCENCE OF PROTEINS INTERACTING WITH NUCLEIC ACIDS. CORRECTION FOR LIGHT ABSORPTION

M. EHRENBERG, E. CRONVALL and R. RIGLER
Institute for Cell Research, Medical Nobel Institute
Karolinska Institutet, S-104 01 Stockholm 60, Sweden

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1. Introduction

The interaction of aminoacyl-tRNA-synthetases with tRNAs has been investigated by observing the change in fluorescence yield of tryptophan residues in the enzyme [1–3]. The change in fluorescence yield cannot be measured directly, since tRNA strongly absorbs at the wavelength at which the tryptophan fluorescence is excited. To account for this light absorption corrections have been applied [1, 3] which are good approximations only at low absorbancies of tRNA and synthetase. We propose a general correction procedure which is not limited to certain experimental conditions. It can be applied to all protein-nucleic acid interactions where the fluorescence of aromatic amino acid residues is modified by the binding process.

2. Material and methods

Adenosine-5′-triphosphate and L-tryptophan were obtained from Sigma, Chemical Company, St. Louis, Mo., USA. The titrations were performed in 0.05 M Tris, pH 7.5.

Fluorescence titrations were performed in an instrument as outlined in fig. 1. Its time resolution and sensitivity permits also the study of relaxation and stopped flow kinetics from changes in quantum yield and polarization of fluorescence [4].

The fluorescence of L-tryptophan was excited at 280 nm and the emission was recorded after passing a WG 320 and UG 11 filter (Schott and Gen.). The pathlength of the cuvettes used was 4.4 mm.

Fluorescence intensity and polarization of tryptophan together with the transmission of the exciting light were recorded when increasing amounts of ATP were added. The polarization was determined as the ratio between the difference and the sum of the light components vibrating parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of polarization of the exciting light beam. To correct for depolarization of the optical system the polarizer was turned at right angle to both components of the emitted light and their intensities were equalized by electronic adjustment. Lamp fluctuations were compensated by a beam splitting arrangement (fig. 1).

For the calculations an IBM 360-75 computer was used.

3. Theory and results

When aminoacyl-tRNA-synthetase is titrated with tRNA the quantum yield φ of the fluorophores (tryptophan) is changed by the binding process. The measured fluorescence F is proportional to the number of quanta/time units reaching the photomultiplier. The proportionality factor connecting F with φ will change during the experiment when a substance absorbing light at the wavelength of excitation is added. Thereby the light along its path in the cuvette will decrease and accordingly the excitation of the fluorophore. This decrease will be a function of the molar extinction coefficient times concentration (k) of the absorbing species. The intensity of the exciting light varies along the x-axis (fig. 2) as

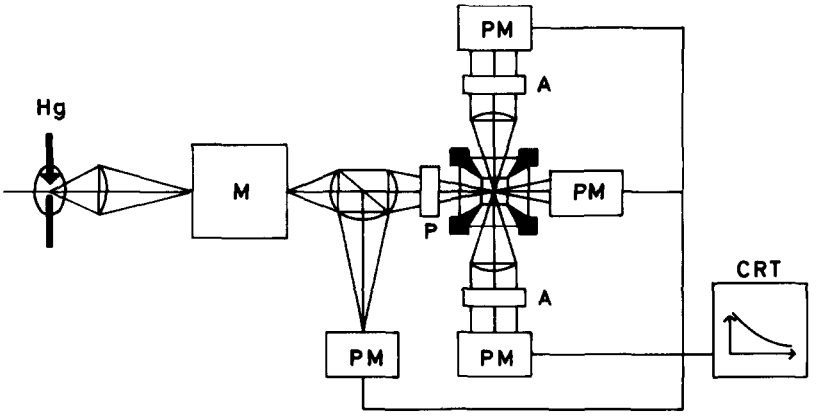

Fig. 1. Scheme of experimental set up for fluorescence measurements. Hg: Mercury arc lamp (Hanovia, 200 W). M: Grating monochromator (Bausch and Lomb). P: Polarizer (Glan-Thompson). A: Analyzers (Polarex Ks-W78, Käsemann). CRT: Oscilloscope or X-Y recorder. PM: Photomultiplier tubes (EMI 9558QA or RCA 1P28).

$$I(x) = I_0 e^{-kx} .$$

The total fluorescence from a segment dx at x is proportional to $I(x)$. The quantity measured will depend on the fraction of the total fluorescence from the segment that reaches the detector.Since different points in the cuvette will see the detector under different solid angles, one has to introduce a weighting factor, which we call $\omega(x)$. The effective fluorescence, $dF(x)$, from a segment dx at x is proportional to $I(x)\omega(x)dx$. That is

$$dF(x) = \text{const.}\ e^{-kx}\omega(x)dx .$$

The effective fluorescence as a function of k from the whole cuvette is then

$$F(k) = \text{const.} \int_0^d e^{-kx}\omega(x)dx$$

where d is the cuvette length. Let k_0 be the absorption of the solution when the experiment starts. To compensate for the decrease in fluorescence caused by increased k-values, the measured fluorescence has to be multiplied by a correction factor, $G(k_0, k)$.

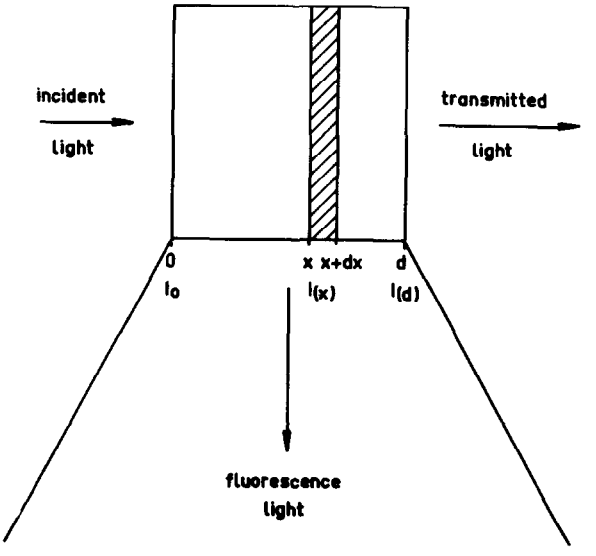

Fig. 2. Scheme of cuvette geometry. Parallel incident light, detector at right angle.

$$G(k_0, k) = \frac{F(k_0)}{F(k)} = \frac{\int_0^d e^{-k_0 x}\omega(x)dx}{\int_0^d e^{-kx}\omega(x)dx}, \quad k \geq k_0$$

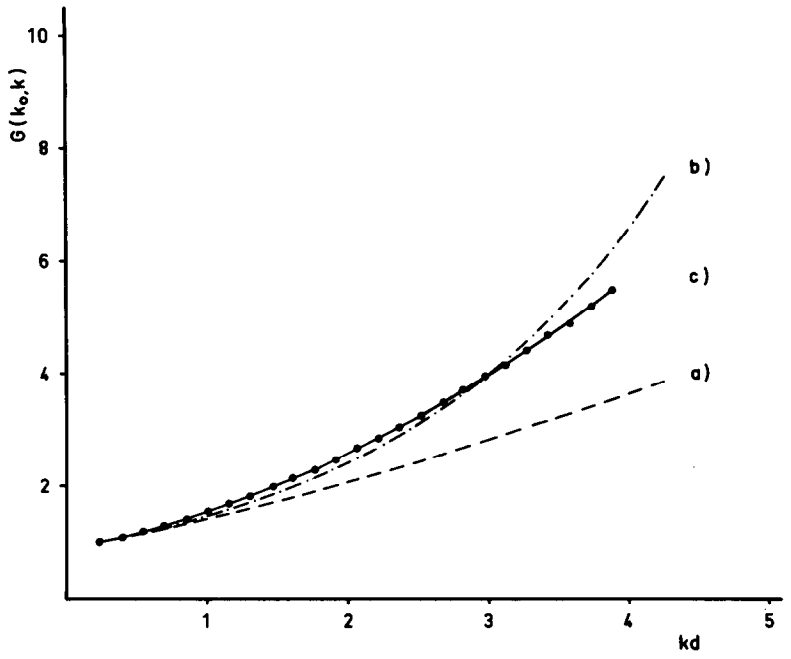

Fig. 3. Correction term $G(k_0, k)$ as a function of kd. (a) $\omega(x)$ = constant over x, (b) $\omega(x)$ = constant at $d/2-\delta < x < d/2+\delta$, $k\delta \ll 1$. (c) experimental points from tryptophan – ATP titration (•). Drawn line: $G(k_0, k)$ calculated from least square fit of $\omega(x)$.

We shall treat three cases separately.

(a) $\omega(x)$ = constant over x,

(b) $\omega(x)$ = constant only over $d/2-\delta < x < d/2+\delta$, where $k\delta \ll 1$, and 0 at all other values of x,

(c) no a priori knowledge of $\omega(x)$.

Thus, when $\omega(x)$ = constant (case a), integration gives:

$$G(k_0, k) = \frac{(1-e^{-k_0 d})}{(1-e^{-kd})} \cdot \frac{k}{k_0}.$$

When $\omega(x)$ is zero (case b), except for a small interval around the middle of the cuvette, one obtains

$$G(k_0, k) = \frac{\int_{d/2-\delta}^{d/2+\delta} e^{-k_0 x}\,dx}{\int_{d/2-\delta}^{d/2+\delta} e^{-kx}\,dx} \approx e^{d/2(k-k_0)}.$$

A direct calculation of $\omega(x)$ starting from the geometry of the experimental set-up is complicated and of no general applicability (case c). Therefore we performed experiments where the fluorescence of tryptophan at a constant concentration was measured as a function of increasing ATP concentrations. If tryptophan and ATP at the concentrations used do not interact, as indicated by measurements of the polarized tryptophan fluorescence in agreement with [5], no change in quantum yield will occur and the weighting factor $\omega(x)$ can be determined from the change in the measured fluorescence intensity as a function of kd. $G(k_0, k)$ is then solved as follows: $\omega(x)$ is expressed as a polynomial in t with unknown coefficients.

$$\omega(t) = \sum_{n=0}^{m} a_n \cdot t^n, \text{ where } t = x/d.$$

We choose the coefficients so that

$$\rho = \int_{k_0}^{k_1} [F(k) - \int_0^1 e^{-ktd}\omega(t)dt]^2 dk \text{ is}$$

minimized, that is

$$\frac{\partial \rho}{\partial a_n} = 0, n = 0,1 ..., m \text{ , and}$$

k_1 is the absorption at the end of the titration. Define

$$g_n(k) = \int_0^1 t^n e^{-ktd} dt,$$

then

$$\rho = \int_{k_0}^{k_1} [F(k) - \sum_{n=0}^{m} a_n \cdot g_n(k)]^2 dk,$$

and

$$0 = \frac{\partial \rho}{\partial a_l} = 2 \int_{k_0}^{k_1} \sum_{n=0}^{m} g_n(k) g_l(k) a_n \, dk -$$

$$2 \int_{k_0}^{k_1} F(k) g_l(k) dk ,$$

$$l = 0,1 ..., m.$$

We obtain now a system of equations to determine the coefficients a_n

$$\sum_{n=0}^{m} b_{ln} a_n = c_l \text{ , } l = 0,1 ....., m$$

with

$$b_{ln} = \int_{k_0}^{k_1} g_l(k) g_n(k) dk, \; l, n = 0,1 ...., m$$

and

$$c_l = \int_{k_0}^{k_1} F(k) g_l(k) dk.$$

The correction factor $G(k_0, k)$ does not account for reabsorption of fluorescence being negligible in our conditions. If necessary an additional correction term has to be introduced.

In fig. 3 $G(k_0/k)$ calculated from the titration of tryptophan with ATP is plotted as a function of kd. The general correction term coincides with the limiting cases (a) and (b) at small kd values. At high kd values correction according to (a) yields too low values of the corrected fluorescence intensity while correction according to (b) results in too high values. Correction (a) has been used by us in a previous communication [3] and is essentially the same as that of Hèléne et al. [1].

In the experiments where aminoacyl-tRNA-synthetases were titrated with tRNA the fluorescence intensity of the enzyme was measured together with the intensity of transmitted light (fig. 2a, c in the following paper [6]). In this way kd is directly measured and changes in the extinction coefficients caused by tRNA-synthetase interaction can be accounted for. Since the applied correction functions demand an exact knowledge of kd particularly at concentrations with low light transmittance, a good linearity of the instrument is essential. The deviation from linearity in our instruments could be described by a constant stray light factor which was determined by a least square fitting of equation

$$kd \text{ measured} = \ln \frac{I_0 + cI_0}{I_0 e^{-k_0 d} + cI_0}$$

where kd = 2.3 A, the corrected absorbance and $I_0 + cI_0$ is the light intensity which is transmitted by pure solvent. In our instrument a value of c = 0.03 was determined. In the titration of synthetases with tRNA, saturation usually was observed already at kd values of 1.4. Although in this interval all discussed correction functions deviate moderately, these differences will change the endpoint of titration and consequently the equilibrium constants calculated from the titration curves. The method described here will be of particular importance, when low equilibrium constants necessitate titrations at high concentrations of the interacting species.

The computer program for the correction procedure is available from the authors on request.

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References

[1] C. Hèléne, F. Brun and M. Yaniv, Biochem. Biophys. Res. Commun. 37 (1969) 393.
[2] C.J. Bruton and B.S. Hartley, J. Mol. Biol. 52 (1970) 165.
[3] R. Rigler, E. Cronvall, R. Hirsch, U. Pachmann and H.G. Zachau, FEBS Letters 11 (1970) 320.
[4] R. Rigler, R. Rabl and T. Jovin, to be published.
[5] J-L. Dimicoli et C. Hèléne, Biochimie 53 (1971) 331.
[6] R. Rigler, E. Cronvall, M. Ehrenberg, U. Pachmann, R. Hirsch and H.G. Zachau, FEBS Letters, this issue.