PHOTOCHEMICAL REACTIONS OF LAC REPRESSOR. EFFECTS ON INDUCER BINDING.

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Summary

Upon U.V. irradiation, lac repressor exhibits a decrease of its fluorescence intensity. Complete quenching is observed when only one of the two tryptophyl residues is modified per protomer. IPTG binding activity decreases with the same kinetics as fluorescence, and this is due to the total inactivation of photodamaged protomers. It is proposed that one tryptophyl residue, the one which is photochemically modified, is involved in the IPTG binding process.

Introduction

The control system of the lac operon in E. coli is a very suitable biological system for physico-chemical studies. The sequences of the lac repressor[1,2] and of lac operator[3] are known, and a great number of results have been obtained from genetic and biochemical studies (see review by Müller-Hill, ref. 4). It is now possible to obtain large amounts of lac repressor[5] and the effects of a large number of effectors (inducers and anti-inducers) have been studied[6,7].

Our experiments are related to the binding of the inducer IPTG (isopropyl β-D-thiogalactoside) to U.V. irradiated lac repressor. Lac repressor contains two tryptophyl residues - 190 and 209 - per protomer. Under native conditions, a blue shift of its fluorescence spectrum is observed upon binding IPTG[8]. In order to investigate the possible role of tryptophan(s) in this binding process, it was desirable to obtain lac repressor molecules whose indole ring(s) is replaced or modified. Using a genetic approach, SOMMER and coworkers[9,10] studied repressor molecules with one of the two tryptophyl residues replaced by tyrosine. We have attempted to modify the lac repressor

by U.V. irradiation. The protein does not contain any S-S group, which has the highest cross section for photodegradation. It could be expected a priori that the major photochemical damage in the protein will be tryptophan degradation, since these residues are the most reactive when light of wavelength longer than 250 nm is used[11].

We have studied the effects of irradiation of lac repressor on fluorescence, circular dichroïsm and IPTG binding, and we have tried to correlate their variations with tryptophan modification.

Materials and Methods

E. coli lac repressor from strain BMH 593 (a gift from Dr. B. MÜLLER-HILL) was purified according to MÜLLER-HILL and coworkers[2]. Concentrations of lac repressor were determined from absorption measurements, assuming that ε_{280} = 21400 per protomer. This value was obtained by microkjehldahl nitrogen analysis, in very good agreement with that calculated from the amino acid composition of the protein.

IPTG (isopropyl β-D-thiogalactoside) was a Sigma product. [^{14}C] IPTG was purchased from CEA, Saclay.

Irradiation experiments were made in a buffer containing 0.2 M potassium phosphate, pH 7.2, and 0.1 mM DTE (dithioerythritol). To avoid aggregate formation occuring upon irradiation, it was necessary to use protein concentrations smaller than 5×10^{-6} M protomer.

IPTG activity was measured by equilibrium dialysis, according to GILBERT and MÜLLER-HILL[13]. TMS II buffer was used : 0.2 M K Cl, 0.01 M Mg acetate, 0.04 M tris/HCl pH 7.5, 0.1 mM EDTA and 0.1 M mercapthoethanol. [^{14}C] IPTG was 10^{-7} M, giving 500 cpm per 100 ml. Three separated samples were dialyzed for each irradiated solution, the dispersion of the results being less than 5 %. Under these conditions, the measured activity is directly proportional to Kxn, where K is the equilibrium constant of the reaction

$$\text{IPTG} + \text{Rep} \rightleftarrows \text{Complex}$$

and n the number of IPTG binding sites per protomer. K and n were determined from equilibrium dialysis data in the irradiation buffer.

Irradiations of lac repressor were performed with an OSRAM HBO 200 watts mercury lamp. A filter MTO H 325 a isolated a band between 250 and 400 nm. Total incident light energy fluence in the wavelength range transmitted by the filter was measured with a KIPP and ZONEN thermopile.

Fluorescence spectra were recorded with a JOBIN-YVON spectrofluorimeter modified to correct for fluctuations in lamp intensity[14]. The absorbance of the samples at the exciting wavelength was smaller than 0.05 to keep the fluorescence intensity proportional to the concentration of fluorescent species.

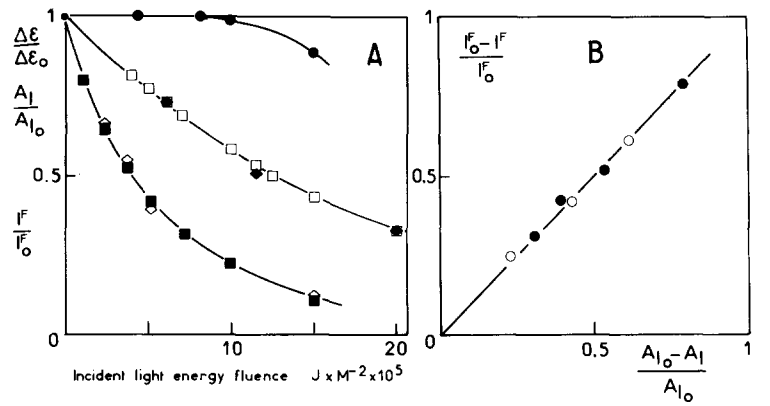


Figure 1 : A. Relative variation with incident light energy fluence, of : upper curve -●-●- : circular dichroïsm intensity at 222 nm ($\Delta\varepsilon/\Delta\varepsilon_o$)
lower curve : fluorescence intensity at 340 nm (λ_{ex} = 280 nm) I^F/I^F_o (-■-■-) and IPTG binding activity A_I/A_{I_o} (-◇-◇-) for lac repressor irradiated alone.
middle curve : fluorescence intensity at 340 nm (λ_{ex} = 280 nm) I^F/I^F_o (-□-□-) and IPTG binding activity A_I/A_{I_o} (-◆-◆-) for lac repressor irradiated in the presence of 10^{-3} M IPTG.

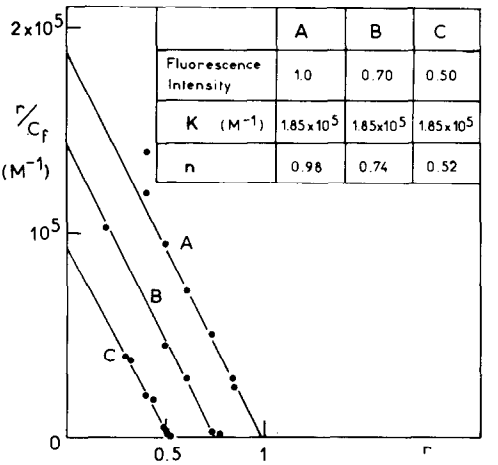
B. Correlation curve $\frac{I^F_o - I^F}{I^F_o} = f\left(\frac{A_{I_o} - A_I}{A_{I_o}}\right)$ between relative fluorescence decrease and relative loss of IPTG binding activity. full circles : lac repressor irradiated alone - open circles : lac repressor irradiated in the presence of 10^{-3} M IPTG.

Circular dichroïsm spectra were recorded at 2°C with a ROUSSEL-JOUAN II apparatus.

Tryptophan concentrations were determined according to a procedure derived from SASAKI[15]. The protein was first digested at pH 8 during 5 hours at 31°C, with trypsin and α-chymotrypsin, both at a final concentration of 0.003 % (w/v). A second proteolytic cleavage with pronase (0.003 % w/v) was performed during 7 hours under the same conditions. Guanidinium chloride was used as denaturing agent, at a final concentration of 5 M. After 30 minutes, the fluorescence spectra were recorded. Above 340 nm, where only tryptophan emits, the fluorescence spectrum of the digested protein is identical to that of free tryptophan. Assays on intact lac repressor gave a number of tryptophan residues per protomer equal to 2 ± 0.1, demonstrating that the procedure is quite suitable for this protein.

Results

Upon U.V. irradiation (250 nm $< \lambda <$ 390 nm), lac repressor exhibited a rapid decrease of its fluorescence intensity



	A	B	C
Fluorescence Intensity	1.0	0.70	0.50
K (M^{-1})	1.85x10^5	1.85x10^5	1.85x10^5
n	0.98	0.74	0.52

Figure 2 : Scatchard plots for the binding of IPTG to lac repressor, r is the fraction of liganded protomers, and C_f is the concentration of free IPTG. The inserted table gives the binding parameters compared with the decrease of fluorescence intensity. A, unirradiated lac repressor, B, irradiated with 2.1 x 10^5 Jm^{-2} (total incident light) C, irradiated with 4 x 10^5 Jm^{-2}.

(fig. 1A). The shape of the fluorescence spectrum was not modified, when excitation wavelength was chosen at wavelength longer than 295 nm. For durations of photolysis longer than 10 minutes (corresponding to about 3x10^5 Joules/m^2 of incident light, and 40 % of loss of fluorescence) a small blue shift of the maximum wavelength of 2 and 4 nm was observed when the fluorescence was excited at 290 and 280 nm respectively.

Long times of irradiation (30 minutes) led to a new broad and weak emission band, with a maximum around 435-440 nm, and an excitation spectrum centered at 345 nm. Nevertheless, this band never interfered with the fluorescence intensity measurements at 345 nm.

IPTG binding activity of the lac repressor (Kxn) also decreased upon irradiation (fig. 1A). The decrease of the fluorescence intensity and that of the IPTG activity are linearly related, as shown on figure 1B. The binding of IPTG to lac repressor leads to a blue shift of 8 nm of the fluorescence spectrum[8]. Similar effects were obser-

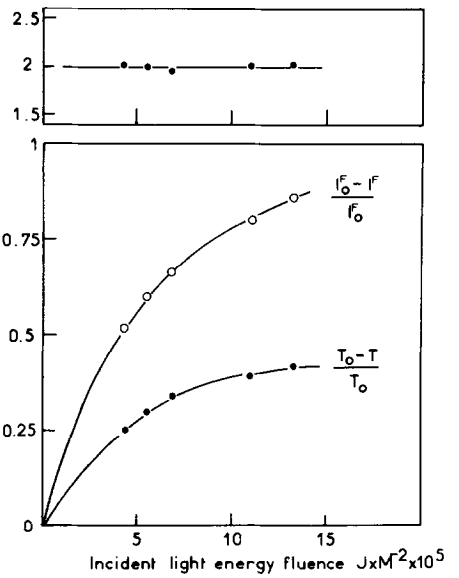


Figure 3 : Relative variation of the decrease of the fluorescence intensity (I^F : fluorescence intensity) and the number of destroyed tryptophan (T : tryptophan content) with incident light energy fluence. The upper part shows the ratio between these two parameters.

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ved with irradiated lac repressor, independent of the duration of irradiation (and therefore of the initial fluorescence loss).

In the presence of saturating concentrations of IPTG, both IPTG binding activity and fluorescence intensity decreased 5 times slower than for unliganded repressor (fig. 1A), which indicates a protective effect of bound IPTG against photochemical damages. However, the linear correlation between the two parameters remains the same as in the absence of IPTG (fig. 1B).

Figure 1A shows the relative variation of the circular dichroïsm intensity of the band characteristic of the peptidic bonds. No change occurs until 75 % of the fluorescence (or IPTG activity) disappears. This indicates that the conformation of the protein is not markedly affected.

Quantitative analysis of the binding process of IPTG to irradiated lac repressor is shown on figure 2. Scatchard plots are straight parallel lines. Binding parameters are listed in figure 2.

The determination of tryptophan content in U. V. irradiated repressor was performed as described in Materials and Methods. Figure 3 shows the relative loss of fluorescence intensity of the irradiated protein, and the relative amount of tryptophan destroyed in the protein. The ratio between these values is equal to two in a large range of irradiation time, demonstrating that the destruction of tryptophyl residues is twice as slow as the decrease of the total fluorescence intensity.

Discussion and Conclusion

In the wavelength range 325-380 nm, the fluorescence of the lac repressor is due to tryptophyl residues. Tyrosines emit weakly at shorter wavelengths, and produce a small shoulder in the spectrum, around 310 nm. The decrease of the fluorescence intensity upon irradiation is due to tryptophan modification (fig. 3). The absence of variation of the CD spectrum excludes that a large conformational change of the protein is responsible for the quenching of tryptophan fluorescence.

Several hypotheses can be put forward to explain that the fraction of modified tryptophyl residues is half that of the relative fluorescence decrease :

(i) only one tryptophyl residue - always the same - is modified in all protomers and the other one does not fluoresce at all, neither in native nor in irradiated protein.

(ii) the modified tryptophyl residue is the same in all protomers and the fluorescence of the other one is quenched either by energy transfer to the photoproduct, or by a local conformational change bringing a quenching group close to the indole ring.

(iii) each tryptophyl residue (190 or 209) has an equal probability of being modified, and the second one no longer emits fluorescence when the first tryptophan is modified, for reasons identical to those given in (ii).

The third hypothesis can be discarded. In this case, three types of protomers should exist : intact ones, protomers with tryptophan 209 modified, and protomers with tryptophan 190 modified. IPTG binding experiments are in disagreement with the existence of

three types of protomers. SOMMER and coworkers[10] found that the two repressors A 190 and A 209, where one tryptophan residue has been replaced by a tyrosine one, keep their binding activity for IPTG with a binding constant equal to that of the wild type for A 190 and 15 times lower for A 209. A mixture of these two species should give non linear Scatchard plots extrapolating to 1. The Scatchard plots presented in figure 2 are linear and extrapolate to a value of n equal to the relative fluorescence intensity. We can thus conclude that only two types of protomers are present in irradiated solutions : protomers unaffected with respect to both fluorescence and IPTG binding properties, and protomers "dead" with respect to these two properties.

It is difficult to choose between hypotheses (i) and (ii). The appearance upon irradiation of a new fluorescent species with an absorption band between 300 and 350 nm makes it possible that fluorescence quenching in each protomer is due to energy transfer from one intact tryptophan to the other modified tryptophan residue. The experimental results presented in figure 3 would require that the transfer probability is equal to unity which in turn requires adequate orientation and close proximity of the two residues. No information being yet available on the tertiary structure of the lac repressor, it is not possible to eliminate or support this assumption.

Hypothesis (i) is in apparent contradiction with the results of SOMMER and coworkers[10] who have built mutants containing only one of the two tryptophans. These authors observed that the two repressors are fluorescent with a yield about half that of the wild type. But in these mutants, a tryptophan is replaced by a tyrosine and it is not obvious that these molecules can be compared with photochemically modified repressor. When the two tryptophans are present energy transfer from one to the other could occur, and the fluorescence of the wild type protein would probably not be the sum of that of the two mutants. Furthermore, replacement of tryptophan by tyrosine may induce a local conformational change which might also modify the fluorescence quantum yield of tryptophyl residues.

We cannot conclude unambiguously on the direct involvement of tryptophan in the IPTG binding process. Tryptophan may interact directly with the sugar, and its photochemical modification may prevent this interaction. Another possibility is that tryptophan does not interact directly with the effector, but its destruction induces a local conformational change, not observed by CD, preventing IPTG binding. Also it cannot be excluded that the decrease of binding activity is due to the photosensitized degradation of another amino acid residue. Such an assumption would imply that this last residue is destroyed with the same rate as tryptophan, suggesting a very efficient photosensitized process.

It has been shown by fluorescence[8], differential absorption[16] and CD measurements[17] that the binding of IPTG to lac repressor modifies the environment of at least one tryptophyl residue. Our results show that the photochemical modification of tryptophan abolishes the ability to bind IPTG, and that the binding of IPTG modifies the rate of the photochemical modification of tryptophan. All these results strongly suggest that one tryptophan is involved, at least indirectly, in the effector binding process.

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