CHLORTETRACYCLINE AS A FLUORESCENT PROBE OF THE FIRST NUCLEOTIDE BINDING SITE OF THE COUPLING FACTOR CF_1 OF SPINACH CHLOROPLASTS

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1. Introduction

Tetracyclines bind divalent cations such as Mg^{2+} or Ca^{2+} forming highly fluorescent complexes [1]. Tetracyclines similarly bind to macromolecules such as ribosomes [2] bovine serum albumin [3] or to membranes [1,4] with a concomitant enhancement of their fluorescence. It is generally thought that tetracyclines bind to macromolecules and membranes by the intermediate action of divalent cations [2] but other mechanisms such as absorption, ionic and hydrophobic binding have also been proposed [5]. Although the binding of tetracycline to membranes is definitely facilitated by divalent cations [6] its binding to protein, e.g., serum albumin, might be mediated by a hydrophobic mechanism [1,5].

The increase of fluorescence which occurred when chlortetracycline was bound to the yeast mitochondrial ATPase complex was reported quenched by addition of ATP or ADP [7]. Possible explanations for these changes are:

1. ADP or ATP might change the polarity of the site where chlortetracycline binds Mg^{2+} or more likely competes with chlortetracycline for binding to the cationic sites of the ATPase. Such a competitive effect has been proposed [6] to explain why addition of ATP or ADP inhibits the uptake of tetracycline by membrane preparations of *Escherichia coli*.
2. ADP or ATP could change the hydrophobicity of a part of the ATPase and hence displace chlortetracycline from this part of the protein or, more generally, change the environment of the sites where the antibiotic remains fixed.

Whatever the way chlortetracycline binds to the ATPase the antibiotic constitutes a good fluorescent probe of the changes induced by the binding of nucleotides to the protein. We have extended its use to the study of the soluble part, CF_1, of the chloroplast ATPase which we have good evidence to think is subjected to conformational changes [8].

Chlortetracycline binds to CF_1 without any intervention of divalent cations. The fluorescence of the antibiotic–protein complex, with a mol/mol stoichiometry, is quenched when the first binding site of CF_1 is occupied by a nucleoside di- or triphosphate. This quenching permitted us to investigate the properties of the first binding site.

2. Materials and methods

CF_1 was prepared as in [8]. No nucleotide was added through all the purification procedure. The total content of nucleotide did not exceed 0.4 mol (AMP–ADP–ATP)/mol protein.

Chlortetracycline–HCl was purchased from Sigma; fresh solutions in water:ethanol, 1 : 1 mixture, were made daily. The concentration was measured by using a molar extinction coefficient at 375 nm $\epsilon_{cm} = 11.8 \times 10^3$ in 0.1 N HCl ethanol [9].

[8-^{14}C]ADP, 48 Ci/mol, was obtained from Schwarz/Mann.

Fluorescence was recorded with an integral spectro-

Abbreviations: AMPPNP, adenilylimidophosphate; εATP, 1,N^6-ethenoadenosine triphosphate; EDTA ethylenediaminetetraacetic acid

fluorimeter Fica MK II. Temperature was kept constant at 20°C. The excitation wavelength was 390 nm and the emission was observed at 520 nm. Chlortetracycline was ~50 μM. At this concentration the excitation at 390 nm is limited by an internal filter effect. The corresponding corrective factor is around 1.7. As there is no significant ΔA_{390} in each separate experiment, this correction factor is always the same for the same total concentration of the antibiotic.

Fixation of [^{14}C]ADP was measured by filtration of the solution through a membrane (Amicon Diaflo XM 50) after the protein was incubated for 30 min in the presence of [^{14}C]ADP. Radioactivity was determined with a liquid scintillation system (Mark III Searle Analytic Inc.).

3. Results

3.1. *Hydrophobic binding of chlortetracycline to CF_1 with a concomitant increase of its fluorescence*

Chlortetracycline in an aqueous medium, free of divalent cations, exhibited a fluorescence intensity dependent on pH (see control in fig.1). The increase of the fluorescence paralleled the progressive dissociation of the C_4-dimethylamine group, characterized by pK_a 7.44 [10]. In the presence of 1 mM ethylenediamine tetraacetate (EDTA) the fluorescence intensity was slightly decreased in alkaline medium.

By mixing CF_1 with an aqueous solution of chlortetracycline, free of divalent cations, we observed an increase of the fluorescence intensity of the antibiotic, completely independent on the presence of EDTA (fig.1). The fluorescence enhancement was maximal around pH 7.

Thus, hydrophobic binding and not ionic binding, dependent on divalent cations, is involved in the formation of this highly fluorescent chlortetracycline–CF_1 complex.

3.2. *A specific part of CF_1 binds 1 mol chlortetracycline/mol protein with a concomitant enhancement of the antibiotic fluorescence*

The fluorescence of a given concentration of chlortetracycline (0.2 μM or 0.5 μM) was measured by adding increasing amounts of the protein (1.5–24 μM). The results are presented on the double reciprocal

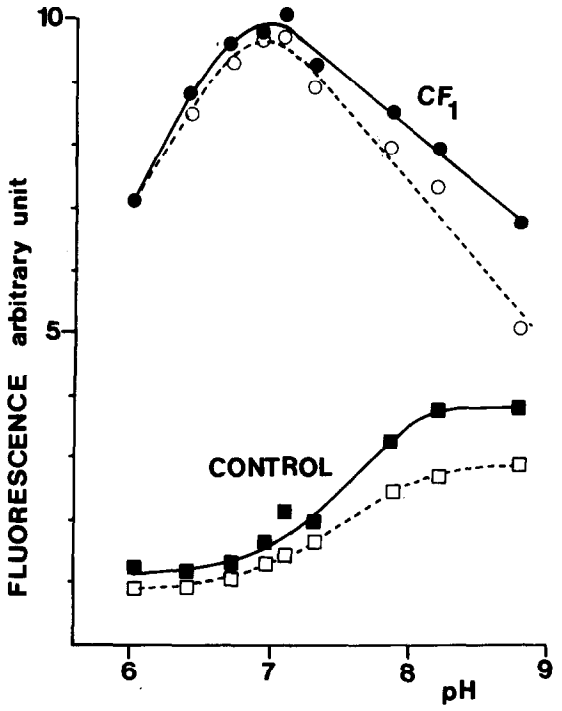

Fig.1. Intensity of the fluorescence of the chlortetracycline, free or bound to CF_1, in the presence or absence of 1 mM EDTA. Medium is 100 mM Tris or Hepes, 50 μM chlortetracycline. Continuous line, in the absence of EDTA; interrupted line, in the presence of 1 mM EDTA. Circles, in the presence of 3 μM CF_1; squares in the absence of CF_1.

plots (fig.2a) from which the enhancement of chlortetracycline fluorescence at infinite protein concentration can be obtained from the extrapolated intercept on the ordinate. The value of this enhancement, relative to 1 μM chlortetracycline, is ΔF_a.

Conversely we measured the chlortetracycline fluorescence when a given amount of CF_1 (0.25 μM or 0.5 μM) was added to increasing concentrations of the antibiotic (below the concentration range where an internal filter effect occurs on the exciting light). By extrapolation of the reciprocal plots (fig.2b) we measured the enhancement of chlortetracycline fluorescence at infinite antibiotic concentration. The value of this enhancement, relative to 1 μM CF_1, is ΔF_p.

The values ΔF_a and ΔF_p are similar. This means that the maximum of the fluorescence enhancement

Fig.2a

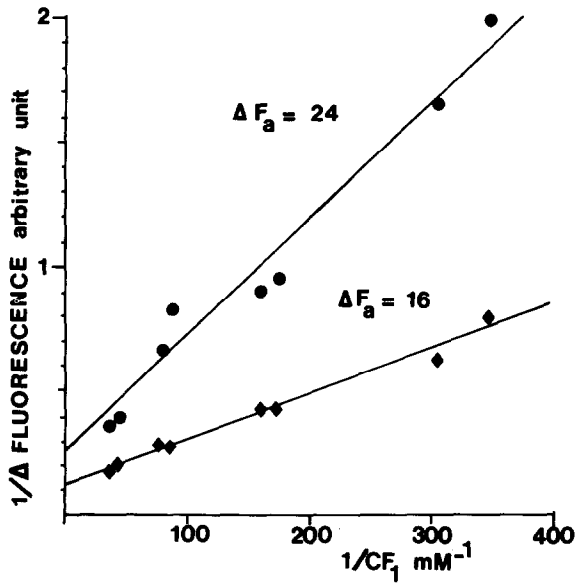

Fig.2b

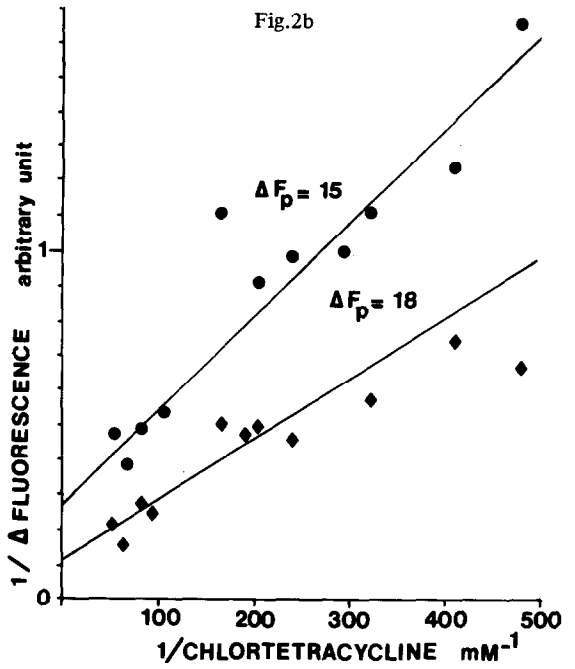

Fig.2. Reciprocal of the fluorescence enhancement as a function of the reciprocal (a) of the CF_1 concentration; (b) of the chlortetracycline concentration. Medium is 100 mM Hepes at pH 6.8. In (a) chlortetracycline is 0.16 μM (upper curve) or 0.52 μM (lower curve); in (b) CF_1 is 0.25 μM (upper curve) or 0.5 μM (lower curve). Enhancement is the increase of the fluorescence intensity observed after mixing CF_1 with chlortetracycline. Final concentrations are as indicated.

corresponds to 1 mol chlortetracycline bound/mol CF_1 in a specific part of the protein.

3.3. *Nucleoside di- and triphosphates reverse the fluorescence enhancement caused by the formation of the chlortetracycline–CF_1 complex*

The enhanced fluorescence which characterized the formation of the chlortetracycline–CF_1 complex may completely reverse to the initial level, observed with the free antibiotic, by addition of ATP or ADP. The presence of EDTA did not change this effect of the nucleotides.

The specificity of the nucleotides (fig.3) for quenching that enhanced fluorescence was the same as that which was observed for photophosphorylations in chloroplasts [11,12]. Nucleoside di- and triphosphates were equally effective. AMP was completely inactive. The activity of the nucleotides depends: (a) on the nature of the base – εADP or εATP were less active than ADP or ATP; (b) on the pyrophosphate bonds – AMPPNP was less effective than ATP,

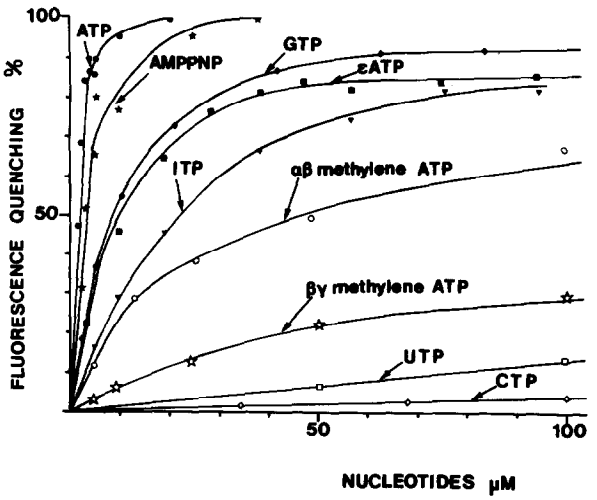

Fig.3. Quenching of the increase of the fluorescence, due to the formation of the chlortetracycline–CF_1 complex, observed after addition of increasing amounts of nucleotides. Medium is 50 mM Tris, 50 μM chlortetracycline and 3 μM CF_1, at pH 8.1.

β–γ methylene ATP less effective than α–β methylene ATP which in turn was less active than ATP (fig.3).

3.4. *The quenching of the fluorescence of the chlortetracycline–CF_1 complex parallels the fixation of ADP on the first binding site*

Only 1 mol chlortetracycline/mol CF_1 is responsible for the fluorescence enchancement during the formation of the antibiotic–CF_1 complex. The reverse of the increase by ADP is the result of a single process which must be the fixation of the nucleotide to 1 of the 3 binding sites of CF_1.

With 3 μM CF_1 the reverse of this increase was almost complete in the presence of 4 μM ADP. At this concentration ~1 mol ADP/mol CF_1 was bound (diamonds in fig.4). So the reverse corresponds to the fixation of ADP on the first binding site of CF_1 and its extent measures the amount of ADP bound to the first site (circles in fig.4).

In fig.4, the interrupted line shows the calculated amount of ADP fixed on both the second and third sites. This amount increased slowly at low concentration of the total ADP and then more quickly when the first binding site was saturated with ADP.

The fixation of ADP was independent of the presence or absence of EDTA. The first binding site exhibited the same properties at pH 8.2 as well as pH 6.8 with K_a ~3 μM^{-1}, as calculated from the Scatchard representation.

The determinations at pH 6.8 are more precise than at pH 8.2 for two reasons.

(1) At pH 6.8, the ratio of the fluorescence of the bound to free chlortetracycline was much higher than at pH 8.2 (fig.1)

(2) Chlortetracycline was more stable at pH 6.8.

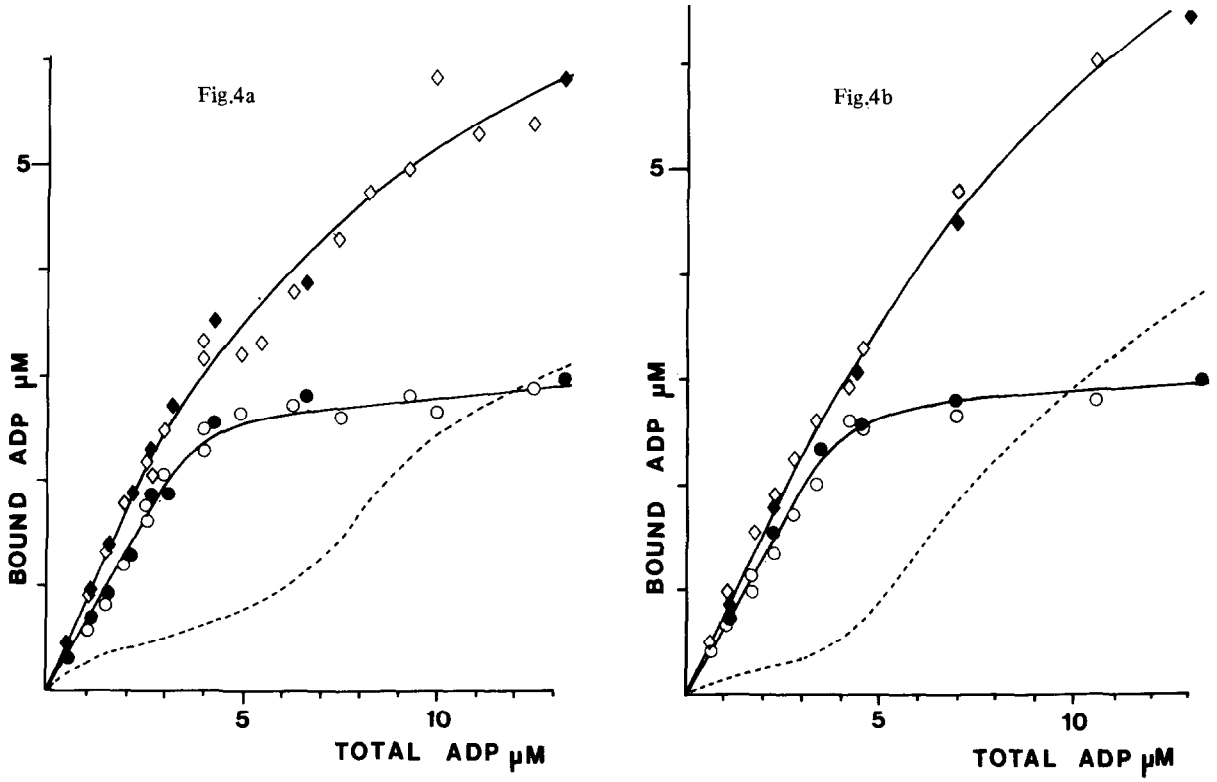

Fig.4. Binding of ADP on CF_1 as a function of the total concentration of ADP. Medium is 50 μM chlortetracycline, 1 mM EDTA when present and (a) at pH 6.8 with 100 mM Hepes or (b) at pH 8.2 with 100 mM Tris. The diamonds represent the total ADP bound to CF_1 and the circles ADP bound on the first binding site of CF_1, in the absence (white symbols) or in the presence of 1 mM EDTA (dark symbols). Interrupted line is the calculated amount of ADP bound to the other sites.

4. Conclusion

The quenching of the fluorescence of the chlortetracycline–CF_1 complex, induced by ADP fixation to the first binding site, indicates a concomitant change of the CF_1 structure which is accompanied by a modification of the hydrophobicity of the part of the protein where chlortetracycline is bound. We already proposed such a conformational change of CF_1 after binding of the first nucleotide molecule [8].

This structural change might induce modifications of the affinity of CF_1 for the nucleotides. This effect should be consistent with the cooperativity between the different sites proposed in [13]. But the amount of ADP bound to the sites other than the first one is known, in our experiments, by difference and with too low a precision to allow the discrimination between models with 3 binding sites independent or interdependent.

The use of chlortetracycline as a probe for the first binding site of CF_1 is only possible in the absence of Mg^{2+}. The fluorescence of chlortetracycline–Mg^{2+} complex is indeed too high with respect to that of the chlortetracycline–CF_1 complex. So we cannot verify whether Mg^{2+} modulate the affinity of CF_1 for the nucleotides as proposed [13].

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