

# The Use of 1-Anilino-8-naphthalene Sulfonate as Fluorescent Probe for Conformational Studies on Ribulose-1,5-bisphosphate Carboxylase

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The influence of  $Mg^{2+}$  ions and temperature on the structure of the enzyme ribulose-1,5-bisphosphate carboxylase was investigated using the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS). The binding of ANS to the enzyme molecule caused a significant increase of fluorescence emission which was further enhanced by the addition of  $Mg^{2+}$ . The temperature dependence of the fluorescence emission indicated a conformational change of the enzyme between 12 and 24 °C. The  $Mg^{2+}$  and the temperature effects were additive. ANS itself did not change the conformation of the enzyme. The influence of the substrates carbon dioxide and ribulose-1,5-bisphosphate, and the effect of the pH of the medium and of a sulfhydryl reducing reagent on fluorescence emission were analysed.

## Introduction

Since the introduction of 1-anilino-8-naphthalene sulfonate (ANS) into protein biochemistry by Weber and Laurence<sup>1</sup>, it has been widely used as a fluorescent probe for conformational changes. This compound undergoes changes in its fluorescent properties as a result of noncovalent interactions with hydrophobic surfaces of proteins<sup>2–4</sup>. The intensity of the emitted fluorescence is proportional to the number of ANS molecules attached to the hydrophobic surface of the enzyme molecule. Intensity changes of the fluorescence can be attributed either to a change in the tertiary or quaternary structure of the enzyme molecule.

Ribulose-1,5-bisphosphate carboxylase (EC. 4.1.1.39), a protein with a molecular weight of 550 000 daltons, is composed of 8 large and 8 small subunits<sup>5</sup>. The subunits differ in their amino acid compositions and the larger ones are more hydrophobic than the smaller ones<sup>6</sup>. Kinetic analyses of ribulose-1,5-bisphosphate carboxylase revealed its allosteric properties<sup>7</sup>. Such enzymes undergo structural changes under the influence of steric effectors, like  $Mg^{2+}$ .

The influence of  $Mg^{2+}$  and the substrates, as well as the pH of the medium, the presence of a sulf-

hydryl reductant, and the temperature on the conformation of the enzyme were investigated.

## Methods and Materials

RuDP carboxylase was isolated from spinach leaves purchased at the local market. The leaves were ground in 100 mM Tris sulfate buffer pH 8.0 containing 10 mM  $MgCl_2$  and 1 mM dithiothreitol and the enzyme was purified by multiple ultrafiltration through an Amicon XM-300 filter and by subsequent centrifugation on a sucrose density gradient (15 to 50% w/v sucrose in the medium mentioned above, 20 h, 70 000  $\times g$ )<sup>8,9</sup>. The fractions were assayed for  $CO_2$  fixation and checked by polyacrylamide gel electrophoresis for purity<sup>8</sup>. Only fractions which showed a single band, characteristic of the  $R_F$ -value of RuDP carboxylase, were pooled. The enzyme preparation was dialyzed against 10 mM Tris sulfate buffer (pH 8.0) and stored below 0 °C.

The enzyme activity was assayed as described previously<sup>8</sup>. The protein content was determined by the method of Lowry<sup>10</sup>. The specific activity was 0.6  $\mu\text{mol/mg protein/min}$ . Polyacrylamide gel electrophoresis was performed as described previously<sup>8</sup>.

Fluorescence was measured with a Zeiss-spectrophotometer PMQ II equipped with the fluorescence attachment ZFM 4 – M4Q III. The exciting light beam (Zeiss H30 DS) was passed through a narrow band-pass filter (365 nm). The intensity of the emitted fluorescence light was measured at 490 nm. The spectra were not corrected for the sensitivity of the photomultiplier. The cuvette contained 2 ml of 10 mM Tris sulfate buffer (pH 8.0) and 0.2 mg of the purified enzyme. All other additions are listed

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Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; RuDP, ribulose-1,5-bisphosphate; Tris, tris-(hydroxymethyl)-aminomethane.



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in the legends to the figures. Experiments were performed under constant temperature conditions and measurements were made 10 min after incubation.

ANS was used either as  $\text{Mg}^{2+}$  salt (Serva, Heidelberg) or as  $\text{NH}_4^+$  salt.

## Results

ANS combines with RuDP carboxylase molecules yielding a fluorescent complex (emitting light 365 nm). The emission spectrum of the ANS saturated enzyme, shown in Fig. 1 reveals that maximal

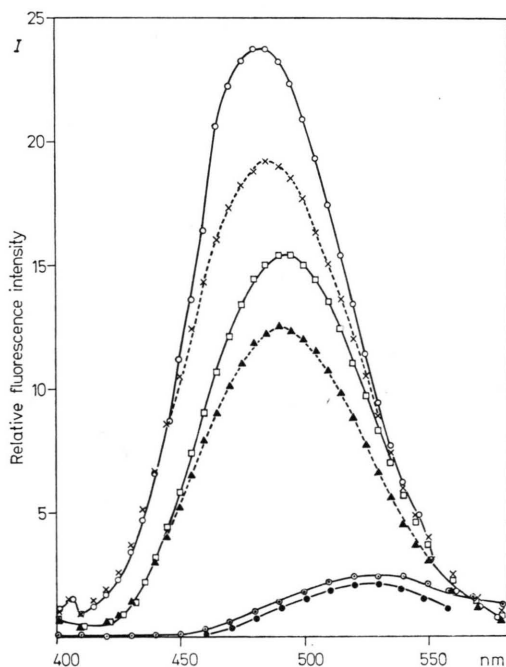


Fig. 1. The emission spectrum of the ANS-protein complex. The fluorescence assay mixture contained in 2 ml 10 mM Tris sulphate buffer pH 8.0, 0.2 mg protein and 125 nmol ANS. The symbols represent:  $\times-\times$ , relative fluorescence intensity recorded at 10 °C;  $\circ-\circ$ , at the same temp. in presence of 0.02 mmol  $\text{MgCl}_2$ ;  $\blacktriangle-\blacktriangle$ , at 30 °C,  $\square-\square$ , at 30 °C in presence of 0.02 mmol  $\text{MgCl}_2$ ;  $\odot-\odot$ , ANS alone at 10 °C;  $\bullet-\bullet$  ANS alone at 30 °C.

fluorescence occurs at about 490 nm. The emission of fluorescence is increased strongly by the addition of  $\text{Mg}^{2+}$  (Table I). Table I shows that temperature, as well, has a remarkable influence on the observed fluorescence. The shift of the peak of the fluorescence emission spectrum was small (5 nm) and therefore all measurements have been carried out at 490 nm. ANS alone or with  $\text{Mg}^{2+}$  showed little fluorescence and the protein solution none at all.

Table I. Influence of substrates,  $\text{Mg}^{2+}$  and DTT on the relative fluorescence intensity.

Additions	Relative fluorescence intensity [%]	
	at 10 °C	at 30 °C
—	100	67
$\text{Mg}^{2+}$	145	93
DTT	93	65
$\text{Mg}^{2+}$ plus DTT	142	95
$\text{HCO}_3^-$	101	66
$\text{HCO}_3^-$ plus $\text{Mg}^{2+}$	129	90
RuDP	95	65
RuDP plus $\text{Mg}^{2+}$	131	88

Using the quantum yield data for the fluorescence of free ANS and of an ANS-protein complex<sup>2</sup>, *i. e.* 0.004 and 0.75 the binding number ( $n$ ) of the ANS-RuDP-carboxylase complex can be estimated by the following formula:

$$\frac{I_{\text{ANS-protein}} - I_{\text{ANS}}}{I_{\text{ANS}}} \frac{QY_{\text{ANS}}}{QY_{\text{ANS-protein}}} \frac{c_{\text{ANS}}}{c_{\text{protein}}} = n,$$

where  $I$  represents the relative fluorescence intensities,  $QY$  the quantum yield values, and  $c$  the concentrations of ANS and protein in the sample. The binding numbers, calculated for the enzyme in the presence and absence of  $\text{Mg}^{2+}$ , at 10 °C are 24 and 19 respectively, and at 30 °C, 15 and 12 respectively.

Fig. 2 shows the results of titrating RuDP carboxylase with increasing amounts of ANS at 10° and 30 °C, plus and minus  $\text{Mg}^{2+}$ . The half saturation of the enzyme molecules occurs at the same ANS concentration in all four cases with a  $K_A$  value of  $2.8 \times 10^{-5}$  M. The straight lines indicate that no positive or negative cooperativity effect is observed in the concentration range between 5 and 100  $\mu\text{M}$  ANS.

A stimulation of the enzyme activity of RuDP carboxylase in the presence of  $\text{Mg}^{2+}$  has been observed previously by different groups<sup>7, 11</sup>. As already shown in Figs 1 and 2,  $\text{Mg}^{2+}$  has a strong influence on the fluorescence of the ANS-enzyme complex. Therefore, the relationship between the  $\text{Mg}^{2+}$  dependence of the carboxylation reaction and the increase in fluorescence was investigated further. The results are summarized in Figs 3 a and b. Increasing amounts of  $\text{Mg}^{2+}$  were added to the enzyme preparation and the change of fluorescence and of the rate of carboxylation reaction were measured. Fig. 3 a shows the  $\text{CO}_2$ -fixation rate as a function of the  $\text{Mg}^{2+}$  concentration (25 °C). The  $K_A$ -value

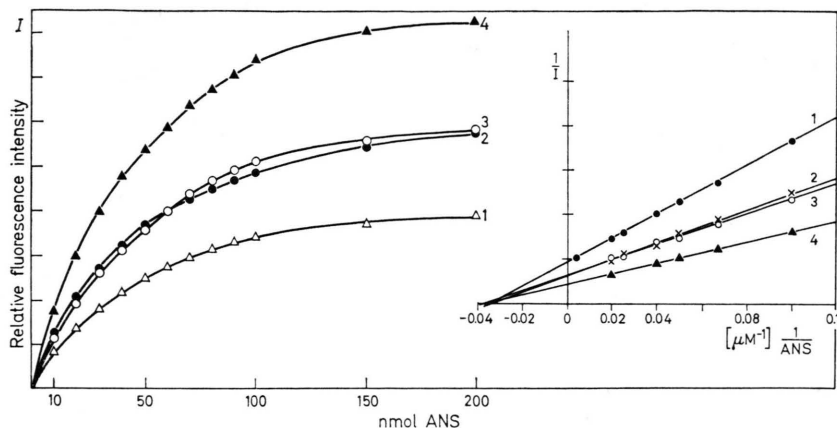


Fig. 2. The titration of the enzyme with ANS. Fluorescence assay mixture as described in the legend to Fig. 1, ANS was added in concentration of 10 nmol per 2  $\mu$ l. Trace 1: measured at 30  $^{\circ}$ C, trace 2: 30  $^{\circ}$ C, plus 10 mM  $\text{Mg}^{2+}$ , trace 3: measured at 10  $^{\circ}$ C, trace 4: 10  $^{\circ}$ C, plus 10 mM  $\text{Mg}^{2+}$ . The insert shows the double reciprocal plot of relative fluorescence intensity vs ANS concentration.

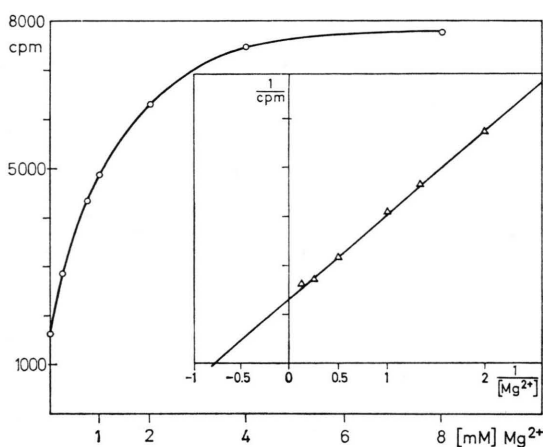


Fig. 3 a. The influence of  $\text{Mg}^{2+}$  on the carboxylation reaction. The assay mixture contained in 0.2 ml : 0.04 mg enzyme protein, 5  $\mu$ mol  $\text{H}^{14}\text{CO}_3^-$  ( $10^6$  cpm/ $\mu$ mol), 1  $\mu$ mol RuDP, 10  $\mu$ mol Tris sulfate buffer pH 8.0, 25  $^{\circ}$ C, after 10 min the reaction was stopped by the addition of 100  $\mu$ l glacial acetic acid;  $\text{Mg}^{2+}$  was added as indicated. The insert shows the double reciprocal plot of CPM vs  $\text{Mg}^{2+}$  concentration.

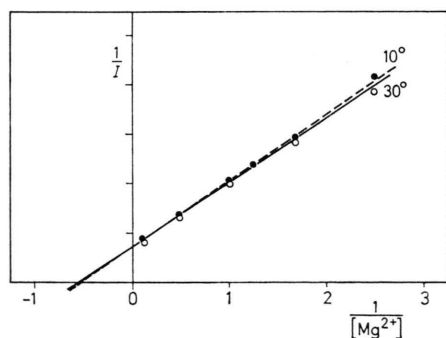


Fig. 3 b. The influence of  $\text{Mg}^{2+}$  on the fluorescence of the ANS-protein complex. Assay conditions as described in the legend to Fig. 1;  $\text{Mg}^{2+}$  was added in concentrations of 200 nmol per 2  $\mu$ l.

for  $\text{Mg}^{2+}$  was determined to be  $1.3 \times 10^{-3}$  M. The titration of the enzyme in presence of an excess amount of ANS as a function of increasing  $\text{Mg}^{2+}$  concentration at 10  $^{\circ}$  and 30  $^{\circ}$ C are presented in Fig. 3 b. At both temperature the  $K_A$ -value for  $\text{Mg}^{2+}$  was calculated to be  $1.7 \times 10^{-3}$  M. The similarity of both  $K_A$ -values may implicate that the bound  $\text{Mg}^{2+}$  which alters the structure of the enzyme molecules by enlarging the hydrophobic area of the protein surface, is also responsible for the increase of the enzyme activity.

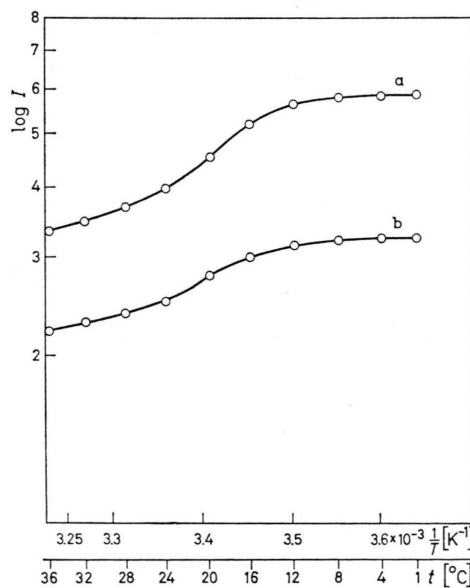


Fig. 4. The temperature dependence of the fluorescence of the ANS-protein complex. The decadic logarithm of fluorescence intensity was plotted against the reciprocal temperature. The fluorescence assay mixture contained in 2 ml 10 mM Tris sulfate buffer pH 8.0, 125 nmol ANS, 0.2 mg protein (curve b) and additional 0.02 mmol  $\text{MgCl}_2$  (curve a). The temperature was controlled in the cuvette before and after the measurements.

Further information on the temperature effect on the enzyme conformation is presented in Fig. 4. The protein solution was temperature-equilibrated in the thermostated cuvette holder at temperatures from 1 to 35 °C, and then the intensity of the emitted light was measured. Fig. 4 may indicate that in the absence of  $Mg^{2+}$  there are two conformations of the enzyme molecules, one at temperatures below 12 °C and one above 24 °C. The sigmoidal shape of the curve could be explained as a change in fluorescence due to an alteration of the protein. In the presence of  $Mg^{2+}$  the same relationship between the reciprocal temperature and the decadic logarithm of the relative fluorescence intensity was maintained. This observation indicates that the presence of  $Mg^{2+}$  does not influence the conformational change by temperature and *vice versa* as shown in Fig. 3b. Furthermore, experiments showed that the observed change is reversible. The kinetics of the alteration follow a first order kinetic (data not shown).

Table I summarizes the effects of the addition of substrates to the ANS-enzyme complex on its fluorescent properties. The additions were made before titration with ANS and samples were measured at two different temperatures (10 and 30 °C). The addition of bicarbonate has no effect on the emission of fluorescence. RuDP decreases the fluorescence compared to the control (which was arbitrarily set as 100% at 10 °C). The fluorescence is increased by  $Mg^{2+}$ , but the addition of bicarbonate or RuDP together with  $Mg^{2+}$  lowers the fluorescence to a level lower than that of  $Mg^{2+}$  alone. This might be explained by the demand for  $Mg^{2+}$  of the substrate binding process. Treatment with the sulfhydryl reducing reagent dithiothreitol has a more pronounced effect at 10 than at 30 °C.

The pH dependence of the fluorescence in the range from 6.5 to 9.5 is presented in Fig. 5. At pH 9.5 the fluorescence diminishes but the emission spectrum of the complex was the same as at pH 8.0 where all measurements so far described have been carried out. The addition of  $Mg^{2+}$  alters the shape of the curves yielding maximal intensities at pH 8.0 (10 °C) and 8.5 (30 °C).

### Discussion

The application of the method of labeling RuDP carboxylase with the fluorescent probe ANS gives insight into possible structural changes of the en-

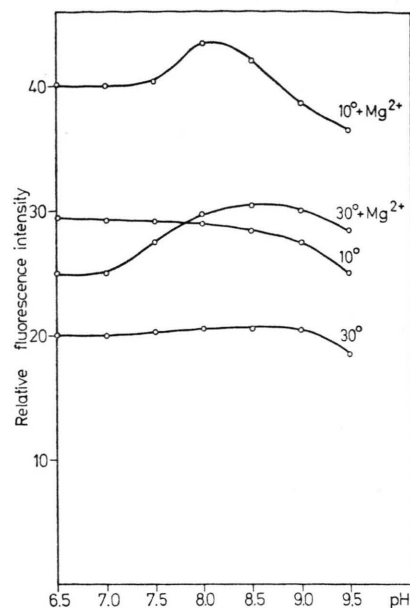


Fig. 5. The pH dependence of the fluorescence of the ANS-protein complex. 10 mM Tris sulfate buffer was adjusted to pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5. The fluorescence assay mixture contained in 2 ml, 125 nmol ANS, 0.2 mg protein, and as indicated 0.02 mmol  $MgCl_2$ .

zyme depending on the assay conditions. However, this information is restricted to the purified enzyme, which has a "high"  $K_m$  value for  $CO_2$ . The "low"  $K_m$  form of the enzyme exist only a short time after chloroplast rupture<sup>12</sup> and so far it has not been possible to isolate and purify the enzyme in this form.

The activity of RuDP carboxylase is stimulated by  $Mg^{2+}$ , which moves from the thylakoids to the stroma in illuminated chloroplasts<sup>13</sup>. The addition of  $Mg^{2+}$  causes a strong increase in the emission of the fluorescent light, indicating that the interaction of the polar groups of the enzyme with the cations may expose more hydrophobic areas of the molecule. The affinity of ANS to the enzyme molecule is not effected, as shown by similar  $K_A$  values in the absence and presence of  $Mg^{2+}$ . These results suggest that  $Mg^{2+}$  does not, per se, affect the binding of ANS, but rather that it increases the number of binding sites, which was also shown by their estimation. Furthermore, the  $K_A$ -values for the binding of  $Mg^{2+}$  to the enzyme, were equal to the  $K_A$  value of  $Mg^{2+}$  in the ANS-enzyme complex as judged by fluorescence analysis.

Another physiological control mechanism of the activity of RuDP carboxylase is the shift of the pH



of the stroma. Dilley and Vernon showed that a light dependent efflux of  $Mg^{2+}$  from the thylakoids accompanies an influx of protons into the membranes<sup>13</sup>. The presence of  $Mg^{2+}$  activates RuDP carboxylase and also shifts the pH optimum of the enzyme from pH 8.5 to lower values<sup>7, 11</sup>. We have therefore studied the dependence of the fluorescent ANS-enzyme complex on the pH of the solution. No change could be observed in the range between 6.5 to 9.0 in the absence of  $Mg^{2+}$ . In the presence of  $Mg^{2+}$  however, the fluorescence of pH 8.0 is increased compared to pH 7.0 and pH 9.5. Since mainly polar amino acids are affected by alterations of the pH, a big effect on the fluorescence would not be expected.

The question, concerning the temperature influence on the conformation of the enzyme was studied by measuring the fluorescence of the ANS-enzyme complex in the temperature range from 1 to 35 °C. The fluorescence changed very little below 12 °C, but decreased on raising the temperature. The sigmoidal shape of the curve in Fig. 4 indicates that between 12 and 24 °C a change of the conformation occurs. The presence of  $Mg^{2+}$  did not alter the shape of the curve. Thus the change of conformation due to the binding of  $Mg^{2+}$  is independent of the alteration of the enzyme due to the influence of the temperature. In this connection, a proposal by Wildman is of great interest, since he suggested that the subunits are more closely packed at room temperature (25 °C) than in the cold (0 °C)<sup>14</sup>. This is in agreement with the temperature effect described in this paper, as the surface of the hydrophobic regions, *i.e.* also the binding number of ANS becomes larger below 12 °C. Pre-

liminary experiments concerning the temperature dependence of the RuDP carboxylase-oxygenase activity also suggest an alteration of the enzyme between 10 and 20 °C (manuscript in preparation).

The effect of bicarbonate and RuDP on the structure of the enzyme was also investigated. The presence of the substrates influenced the emission of fluorescence of the ANS-enzyme complex although these effects were not as large as those of  $Mg^{2+}$ . Recently Kwok and Wildman showed that RuDP altered the conformation of RuDP carboxylase<sup>15</sup>. This observation, based on difference absorption spectroscopic studies, revealed that both tyrosyl and tryptophyl residues were translocated to a more polar environment.

A curiosity of RuDP carboxylase is the high content of half cystine residues of 96 per mole enzyme. The cysteine residues play a major role in the binding of the substrates and are an integral part of the catalytic sites<sup>16</sup>. Treatment of the enzyme with dithiothreitol has only a small effect on the binding of the ANS molecules.

RuDP carboxylase is also stimulated by white fluorescent light<sup>8, 17</sup>. The question of whether the light activation also causes a change of the conformation of the enzyme could not be resolved because the activating light beam interfered with the fluorescence measurement. It should be noticed, however, that the activation of the enzyme by light could only be observed in the presence of  $Mg^{2+}$ .

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