Volume 284, number 1, 23–26 FEBS 09794 June 1991
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ADONIS 001457939100502B

Activation-dependent changes in microenvironment of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase

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Received 28 February 1991

The spinach ribulose 1,5-bisphosphate carboxylase/oxygenase was labelled with *o*-phthalaldehyde, which forms a stable fluorescent isoindole adduct at the active site. The fluorescence behaviour of the labelled enzyme after activation to different levels by Mg^{2+} was compared with that of a synthetic isoindole adduct of *o*-phthalaldehyde, namely 1-(hydroxyethylthio)-2-β hydroxyethylisoindole in solvents of different pH and polarity. The results suggest that the microenvironment at the catalytically incompetent active site of the unactivated Rubisco is highly acidic (pH <2) in nature. The activation by Mg^{2+} results in the conformational change such that the effective pH at the active site increases to > 8. The polarity of the active site of the activated enzyme was found to be similar to that of a mixture of hexane and toluene.

Ribulose bisphophate carboxylase; Activation; *o*-Phthalaldehyde; Fluorescence

1. INTRODUCTION

The ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the key enzyme of the Calvin cycle. Rubisco from spinach consists of 8 large subunits (58000 Da) contributing to 8 active sites and 8 small units (14000 Da), whose function is not clearly understood, even though it has been shown to be essential for the activity of this hexadecameric protein. The enzyme requires the formation of a ternary complex of Rubisco-CO_2-Mg^{2+} [1] for its catalytic activity. A number of attempts based on spectroscopic, chemical and X-ray crystallographic techniques have been made to study the conformational change in Rubisco molecule on activation with Mg^{2+} and CO_2 [2–6]. These studies qualitatively describe the overall changes in the molecular conformation of Rubisco but fail to shed light on the changes brought about by activation in the structure of the active site. It is important to study the activation dependent changes at the active site because of their direct involvement in promoting catalytic activity of the enzyme. A conformational change at the active site (i.e. a change in the organization of different amino acid residues present at the active site) manifests itself as a change in its microenvironment. Therefore, in the present study, we have investigated the nature of the microenvironment at the active site of Rubisco both before and after activation by Mg^{2+}. The enzyme was labelled with *o*-phthalaldehyde (OPA) which forms covalent bonds with the SH group of cysteine and the NH group of lysine at the active site resulting in a strongly fluorescent stable isoindole adduct [7,8]. Activation by Mg^{2+} had a marked effect on the fluorescence emission of the Rubisco-OPA adduct. An isoindole derivative 1-(β-hydroxyethylthio)-2-β hydroxyethylisoindole (EA adduct) of OPA was synthesised by the method of Simons et al. [9]. The fluorescence properties of this model compound were studied in the solvents of different pH and polarity. These properties were utilised to interpret the fluorescence behaviour of the Rubisco-OPA adduct.

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2. MATERIALS AND METHODS

Rubisco was purified to homogeneity from spinach leaves according to the method of Paulsen and Lane [10]. Protein concentration was estimated by measuring the absorbance at 280 nm assuming an $E^{1}\%\ cm^{-1} = 16.4$ [11]. The carboxylase activity of the enzyme was assayed according to the method of Lorimer et al. [12]. Labelling of Rubisco with OPA was carried out as described by Bhagwat and Gopalakrishna [7]. The concentration of OPA-Rubisco adduct was estimated by measuring the absorbance at 337 nm using the molar extinction coefficient of 7.66 $mM^{-1}\ cm^{-1}$ [9]. The isoindole derivative 1-(β-hydroxyethylthio)-2-β hydroxyethylisoindole of OPA was synthesised by the method of Simon and Johnson [9]. Steady state fluorescence measurements were carried out on an Hitachi F 3010 fluorescence spectrometer. The fluorescence life times were estimated on single photon counting fluorescence spectrometer SP-70 (Applied Photophysics UK). The fluorescence decay profiles were analysed by iterating χ^2 fitting [13]. The goodness of fit was judged by weighted residuals and reduced χ^2 values.

3. RESULTS

3.1. *Fluorescence from OPA labelled Rubisco*

To investigate the effect of activation by Mg^{2+} on the microenvironment at the active site both OPA labelled

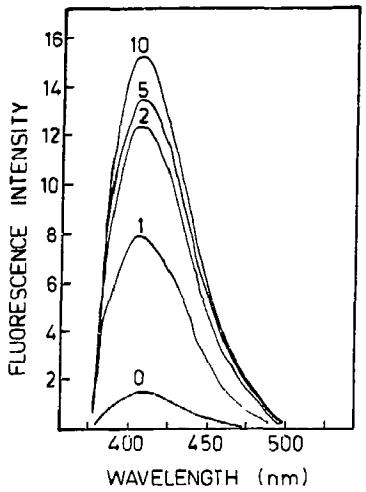

Fig. 1. Effect of activation by different concentrations of Mg^{2+} (mM) as indicated in the figure, on the fluorescence emission spectrum of Rubisco-OPA adduct (excitation 337 nm).

and unlabelled Rubisco samples were extensively dialysed against borate buffer (50 mM, pH 8.0) containing different concentrations of Mg^{2+}. While the labelled samples were used for fluorescence study, the unlabelled samples were used to estimate the carboxylase activity at different Mg^{2+} concentrations. The fluorescence of Rubisco-OPA adduct was studied by exciting the OPA labelled enzyme at 337 nm. At this wavelength the light is predominantly absorbed by the OPA-isoindole adduct. The fluorescence spectrum showed a single line with a peak at 406 nm. The position of the maximum was not altered by Mg^{2+} concentration. However, as shown in Fig. 1, the fluorescence intensity of the adduct increased by an order of magnitude when the Mg^{2+} concentration increased from 0 to 10 mM. Furthermore, the fluorescence intensity of Rubisco-OPA adduct had a direct relationship with the carboxylase activity measured in unlabelled enzyme at different Mg^{2+} concentrations as shown in Fig. 2.

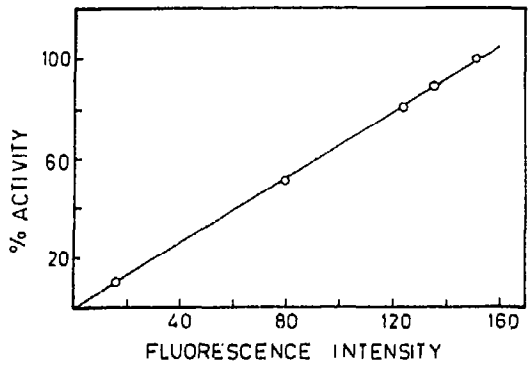

Fig. 2. Relationship between the percent carboxylase activity and fluorescence intensity of OPA-labelled Rubisco after activation with different concentrations of Mg^{2+}. The activity corresponding to activation at 10 mM Mg^{2+} has been assumed as 100%.

Table I

Effect of activation by Mg^{2+} on the fluorescence life time at 410 nm; excitation wavelength 330 nm

Mg^{2+} (mM)	A_1	τ_1 (ns)	A_2	τ_2 (ns)	τ_{AV}*
0.0	0.44	1.99	0.10	9.77	3.43
1.0	0.43	1.98	0.09	10.31	3.50
2.0	0.43	2.13	0.10	10.44	3.63
5.0	0.44	2.33	0.11	10.64	3.99
10.0	0.49	1.77	0.15	11.30	4.00

* $\tau_{AV} = (A_1\tau_1 + A_2\tau_2)/(A_1 + A_2)$

The fluorescence decay of the Rubisco-OPA adduct at 406 nm (λ_{ex} 337) was found to be nonexponential (Table I). The data could be fit well with the expression given below

$$I(t) = A_1e^{-}t/\tau_1 + A_2e^{-}t/\tau_2$$

Here, $I(t)$ is fluorescence intensity at time t and A_1, A_2 and τ_1, τ_2 are the proportionality constant and the exponential life times of the two decay components respectively. Table I shows the estimated values for A_1, A_2 and τ_1, τ_2 for the samples activated at different Mg^{2+} concentrations. The average life time [$\tau av = (A_1\tau_1 + A_2\tau_2)/(A_1 + A_2)$] was found to be 3.43 ns for 0 mM Mg^{2+} which increased to 4.0 ns for 10 mM Mg^{2+}.

3.2. *Fluorescence from synthetic EA adduct*

3.2.1. Effect of solvent pH

The synthetic EA adduct was dissolved in aqueous solution of pH ranging from 2 to 10. The fluorescence spectra recorded at different pH with excitation at 337 nm are shown in Fig. 3. The fluorescence intensity

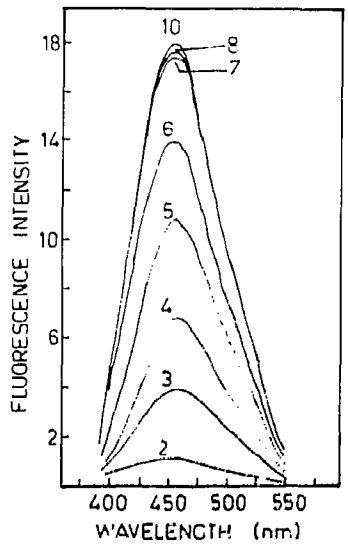

Fig. 3. Effect of solvent pH, as indicated in the figure, on the fluorescence intensity of the EA adduct (ex. = 337 nm).

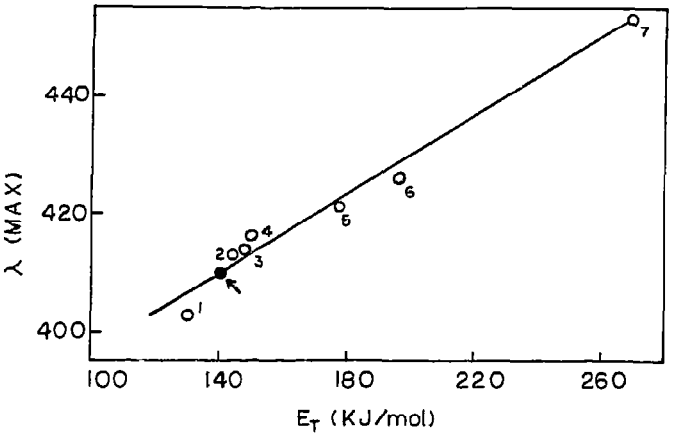

Fig. 4. Relationship between λ_{max} fluorescence (ex = 337 nm) of EA adduct and betain iodide transition energies (E_T) found in different solvents. The solvents used are: 1, hexane; 2, toluene; 3, benzene; 4, dioxane; 5, acetonitrile; 6, acetone; 7, water. The λ_{max} for Rubisco-OPA adduct is indicated by the arrow.

increased by an order of magnitude when the pH was changed from 2 to 7. Further increase in pH did not have any effect on the fluorescence intensity. The pH of all the samples was then adjusted to 10 by adding a small amount of 1 M NaOH and the fluorescence spectra were recorded again. The intensity of fluorescence in all the samples was found to be the same (within ±2%) after correcting for dilution (data not included).

3.2.2. Effect of solvent polarity

The synthetic EA adduct was dissolved in the solvents of different polarity and its fluorescence properties were analysed. Solvents with higher polarity caused bathochromic red shift in the fluorescence maxima. In Fig. 4, we have plotted the fluorescence (λ_{em}) against betain iodide transition energies (E_T) in different solvents [9]. The behaviour was similar to that reported by Palczewski et al. [14]. The position of fluorescence maximum for Rubisco-OPA adduct is shown by the arrow.

4. DISCUSSION

Studies on the synthetic (EA) adduct show that the fluorescence properties of thioisoindole adduct of OPA are very sensitive to its environment. While the EA adduct shows strong fluorescence in solutions of pH > 7, its intensity is very much reduced in acidic medium (Fig. 3). At pH < 2, the fluorescence of the adduct is almost completely quenched. The effect of pH on the fluorescence is, however, found to be reversible, since its intensity is completely restored by increasing the pH of the acidic solution to 10. This large effect of pH on the fluorescence intensity of the adduct can be explained on the basis of the interactions between the aromatic ring of the adduct with water protons. There is considerable evidence that the centre of an aromatic ring can act as a hydrogen bond acceptor for a hydrogen bond donor (like -O-H-, >N-H) [15–17]. These interactions are pH dependent and would, therefore, affect the fluorescence intensity of EA adduct according to pH of the solution. At pH 2 or less these hydrogen bond-like interactions may very effectively quench the EA adduct fluorescence. The polarity of the solvent affects the position of the emission maximum of the adduct fluorescence (Fig. 4). Polar solvents cause bathochromic red shift in the emission maximum. These properties can be utilised to analyse the fluorescence behaviour of the Rubisco-OPA adduct and as a consequence shed some light on the microenvironment at the active site of Rubisco.

The fluorescence intensity of Rubisco adduct increases by about an order of magnitude when the Mg^{2+} concentration is increased from 0 to 10 mM (Fig. 1). This is associated with an increase in the average life time of about 15% (Table I), which suggests only a marginal increase in the quantum yield of the fluorophor. Since the concentration of Rubisco-OPA adduct was the same in all the samples, the increase in the fluorescence intensity of Rubisco-OPA adduct with Mg^{2+} concentration suggests that not all the enzyme-OPA adduct in the sample fluoresce and that the number of fluorescent adducts increase with increase in Mg^{2+} concentration. Since increasing Mg^{2+} concentration increases the number of catalytically competent active sites, it can be argued that the OPA adduct at the active site of Rubisco fluoresces only when the site has been activated by Mg^{2+}. This argument would imply that the fluorescence intensity of the Rubisco-OPA adduct should have the same dependence on Mg^{2+} concentration as the catalytic activity of the unlabelled enzyme. This is consistent with the fact that the graph drawn for percent activity against fluorescence intensity of the labelled enzyme shows a straight line (Fig. 2). A comparison of these results with that of EA adduct gives us an qualitative estimate of the pH of the active site both before and after activation. The complete quenching of fluorescence of the adduct in the absence of Mg^{2+} suggests that the effective pH of the active site before activation is likely to be around 2. The activation by Mg^{2+} brings about sufficient conformational change at the active site to increase the effective pH to 7 or more to make the isoindole adduct strongly fluorescent. A number of lysine and arginine residues having positively charged functional groups are known to be present in the vicinity of the active site [18]. Displacement of some of these amino acids (as a result of activation by Mg^{2+}) closer to the active site will increase its effective pH. The emission maximum of the fluorescence from OPA labelled Rubisco at 406 nm corresponds to the λ_{max} of EA adduct in a mixture of hexane and toluene (Fig. 4). The position of the fluorescence maximum is not affected by the level of activation by Mg^{2+}.

This is expected because (as we have shown) only those isoindole adducts in Rubisco fluoresce which are at the catalytically competent site. Therefore, it can be concluded from our studies that the microenvironment of the catalytically competent active site is highly nonpolar and basic in nature. The increase in the positive charge at the active site as a result of activation by Mg^{2+} possibly stabilizes the transition state and/or facilitates subsequent catalysis.

Acknowledgements: The authors gratefully acknowledge Dr K.K. Kannan for his critical comments and suggestions.

REFERENCES

[1] Holzenburg, A. and Mayer, F. (1989) Electron Microsc. Rev. 2, 139–169.
[2] Vater, J., Salnikow, J. and Kleinkauf, H. (1977) Biochem. Biophys. Res. Commun. 74, 1618–1625.
[3] Grebanier, A.E., Champagne, D. and Roy, H. (1985) Biochemistry 17, 5150–5155.
[4] Pilz, I., Schwarz, E., Pal, G.P. and Saenger, W. (1987) Zeitschr. Naturforsch. 42, 1089–1091.
[5] Verma, N.C. and Bhagwat, A.S. (1985) Biochem. Biophys. Res. Commun. 129, 256–261.
[6] Bhagwat, A.S. and Verma, N.C. (1988) Ind. Biochem. Biophys. 25, 244–248.
[7] Bhagwat, A.S. and Gopala Krishna, K. (1986) Biochim. Biophys. Acta 873, 45–52.
[8] Dimroth, K., Reichardt, C., Siepmann, T. and Bohlmann, F. (1963) Ann. Chim 661, 1–37.
[9] Simons, S.S. and Johnson, D.F. (1978) J. Org. Chem. 43, 2886–2891.
[10] Paulsen, J.M. and Lane, M.D. (1966) Biochemistry 5, 2350–2357.
[11] Wishnick, M. and Lane, M.D. (1971) Methods Enzymol. 23, 572–577.
[12] Lorimer, G.H., Badger, M.A. and Andrews, T.J. (1977) Anal. Biochem. 78, 66–75.
[13] O'Connor, D.V., Ware, W.R. and Andre, J.C. (1979) J. Phys. Chem. 83, 1333–1343.
[14] Palczewski, K., Hargrave, P.A. and Kochman, M. (1983) Eur. J. Biochem. 137, 429–435.
[15] Levitt, M. and Perutz, M.F. (1988) J. Mol. Biol. 201, 751–754.
[16] Burley, S.K. and Petsko, G.A. (1986) FEBS Lett. 203, 139–143.
[17] Tuehsen, E. and Woodwark, C. (1987) Biochemistry 26, 1918–1925.
[18] Incharoensakdi, A. and Takabe, T. (1987) J. Sci. Soc. Thailand 13, 133–157.