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ENERGY TRANSFER FROM TRYPTOPHAN RESIDUES TO PYRIDOXAL 5'-PHOSPHATE AT THE ACTIVE SITE OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

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Forster's mechanism of radiationless energy transfer has been used to estimate average distance between tryptophan residues and pyridoxal 5'-phosphate bound at the active site of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase. This distance was found to depend on the activity of the enzyme and was 29 Å for a freshly purified enzyme (activity 1.7 μ moles CO_2 fixed/min/mg protein) and 37 Å for a 6 week old enzyme stored at 4°C (activity 0.07 μ moles CO_2 fixed/min/mg protein).

Pyridoxal 5'-phosphate (PLP) inactivates ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) by preferentially binding to ϵ -amino groups of essential lysyl residues at the active site (1-3). In the spinach enzyme, there are 16 lysyl residues which are essential for enzyme activity. The Schiff-base formed between PLP and lysyl residue is stabilized on reduction with $NaBH_4$ (3). This covalently bound PLP after reduction (here onwards called Lys-PMP) has characteristic spectral properties which have been exploited to study the events at the active centre of RuBisCO (4). The absorption maximum of Lys-PMP at 325 nm overlaps very well with the intrinsic fluorescence spectrum of the native enzyme, which is dominated by the emission from tryptophan (Trp) residues. On

Abbreviations: PLP, pyridoxal 5'-phosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; Lys-PMP, reduced lysine-PLP complex; Trp, tryptophan.

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excitation at 325 nm, Lys-PMP gives an emission spectrum having a peak at 388 nm (5). Since the absorption of Lys-PMP is almost negligible in the region of 270-290 nm, where Trp absorbs , this provides an unique opportunity to study the energy transfer from Trp residues to Lys-PMP at the active site. In this report we have used Forster dipole-dipole interactions to estimate the average distance between Trp residues and PLP bound at the active site (6). We have specifically studied the effect of the activation state of the enzyme on this distance.

METHODS AND MATERIALS:

RuBisCO from spinach leaves was purified to homogeneity according to the method of Paulsen and Lane (7). Protein concentration was estimated by measuring the absorbance at 280 nm by assuming an $E^{1\%}_{1cm} = 16.4$ (8). Activation and assay of the carboxylase activity was done as described by Lorimer et al. (9). The specific activity of the freshly purified and activated enzyme was 1.7 μ moles CO_2 fixed/min/mg protein.

Binding of PLP at the active site of RuBisCO and reduction of Schiff-base was carried out in Bicine-KOH buffer (pH 8.0) containing 1 mM dithiothreitol and 0.2 mM EDTA by the method described in reference 3. The PLP concentration was varied from 10 μM to 1 mM to obtain different bound PLP-enzyme molar ratios, which were estimated by comparing the absorbance at 325 nm and 270 nm. The molar extinction coefficient of N^6-phosphopyridoxal lysine at 325 nm was taken to be 4800 M^{-1} cm^{-1} (3). All fluorescence measurements were done on SP-70A fluorescence spectrometer (Applied Photophysics, U.K.).

RESULTS AND DISCUSSION:

The intrinsic fluorescence from RuBisCO when excited at 290 nm, showed a peak at 336 nm (Fig.1) and was essentially the same as that from tryptophan solution (not shown in the figure). Activation of the enzyme with metal ions and/or binding of ribulose-1,5-bisphosphate (RuBP) had no effect on this fluorescence. This is consistent with the earlier studies (10). When PLP was bound at the active site of the freshly purified and activated (with HCO_3^- and Mg^{2+}) enzyme, the peak at 336 nm

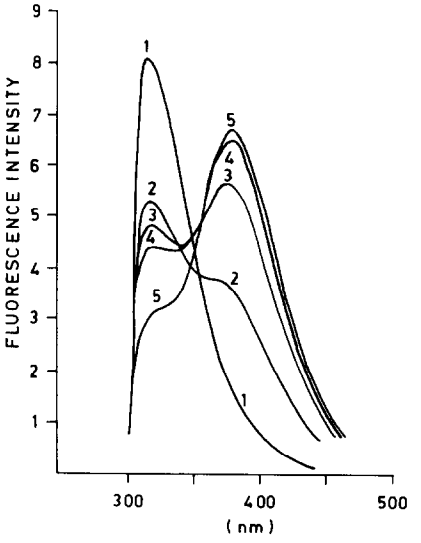

Fig.1 Fluorescence emission spectrum of freshly purified and activated RuBisCO (10 mM Mg^{2+}, 20 mM HCO_3^-) with (1) nil (2) 20 μM (3) 50 μM (4) 150 μM and (5) 500 μM PLP. Excitation at 290 nm.

(here onwards called peak I) decreased in intensity and another peak at 388 nm (here onwards called peak II) corresponding to Lys-PMP emission appeared. Since Lys-PMP does not absorb at the excitation wavelength (290 nm) used, the appearance of peak II indicates an efficient energy transfer from Trp residues to PLP bound at the active site of RuBisCO. The intensity of peak II increased with PLP concentration reaching a maximum at 150 μM PLP. A further increase in PLP concentration did not significantly affect peak II, even though a decrease in peak I was noted (curve 4, Fig.1). The saturation behaviour of peak II indicates that at low concentrations ($< 150\,\mu$M) PLP binds specifically at the active site only, where it has a reasonable fluorescence quantum yield. However, at higher concentrations ($> 150\,\mu$M) PLP is attached to other than active sites, where its fluorescence is strongly quenched. These findings are consistent with the result of Vater et al (4). The fact that peak I continued to decrease even after peak II levelled

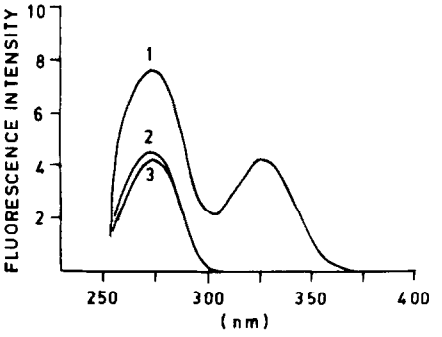

Fig.2 Excitation spectrum of (1) freshly purified and activated RuBisCO with 100 μM PLP; emission at 405 nm (2) N-acetyl tryptophanamide; emission at 340 nm and (3) freshly purified and activated RuBisCO without PLP; emission at 340 nm.

off indicates that the energy transfer from Trp residues to PLP attached to other sites is also efficient. The excitation spectrum of RuBisCO-PLP complex was found to be identical with that of N-acetyltryptophanamide in the UV region (Fig.2). This shows that the light absorbed by Trp residues was responsible for the observed emission from the enzyme bound PLP.

To study the effect of activation state of the enzyme on this energy transfer, we inactivated the enzyme by storing it at 4°C for a period of 6 weeks. After this period the enzyme was treated with bicarbonate and Mg^{2+} by the method described in reference 9 and the residual carboxylase activity was measured to be 0.07 μ moles CO_2 fixed/min/mg protein. The enzyme was treated with 50 μM of PLP to form Lys-PMP complex at the active site. The emission spectra of this sample and that of a freshly purified enzyme with same treatment with bicarbonate, Mg^{2+} and PLP, when excited at 290 nm are shown in Fig.3 (curves 2 and 3 respectively). As can be seen from the figure that both decrease in peak I and increase in the fluorescence intensity at the position of peak II are much less in the case of inactive (stored) enzyme in comparison to that found in the case of ac-

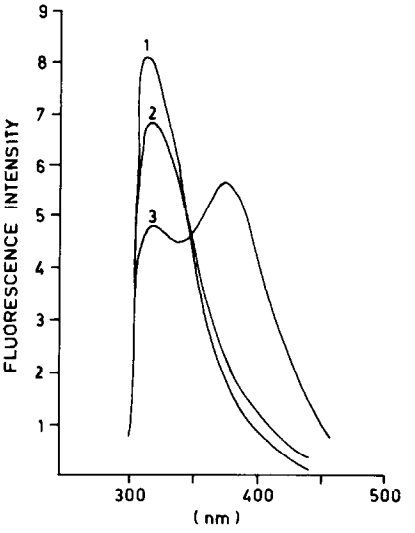

Fig.3 Fluorescence emission spectrum of (1) freshly purified and activated RuBisCO without PLP (2) 6 week old activated RuBisCO with 50 μM PLP and (3) freshly purified and activated RuBisCO with 50 μM PLP. Excitation at 290 nm.

tive (fresh) enzyme. The molar ratio of bound PLP/enzyme molecule was found to be same in both active and inactive enzyme. Therefore, the difference in the efficiency of the energy transfer in two cases can be attributed to the difference in the enzymatic activity only. Assuming that this energy transfer is due to weak dipole-dipole interactions (Forster's mechanism), then the fractional energy transfer efficiency E is given by (6)

$$E = R_o^6 / (R_o^6 + R^6) \qquad (1)$$

Where R is the distance between donor (Trp) and acceptor (PLP) and Ro is the characteristic distance (R for E = 0.5) and was computed to be 28 A° for this system using the method described in reference 6. For this purpose a random orientation of the donor-acceptor transition dipoles was assumed. Efficiency of the energy transfer was estimated from the quenching of Trp fluorescence. The distance R thus computed from equation (1) was found to be 29 Å for fresh (active) and 37 Å for stored

(inactive) enzyme. Although, it is difficult at this stage to identify which of the Trp residues present in the enzyme are responsible for the energy transfer to PLP at the active site, our results indicate that a definite conformation, which facilitates this energy transfer, is essential for the carboxylase activity of RuBisCO.

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