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A FLUORIMETRIC DIFFERENTIATION OF THE BINDING SITES OF THE D-RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM SPINACH FOR PYRIDOXAL 5'-PHOSPHATE

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SUMMARY:

The D-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach possesses multiple binding sites for pyridoxal 5'-phosphate, a specific reagent for reactive amino groups of the enzyme. Pyridoxal 5'-phosphate binding can be differentiated fluorimetrically. A high affinity site of the carboxylase for this inhibitor has been selectively detected and characterized. Our results are consistent with the hypothesis that pyridoxal 5'-phosphate functions as an active site directed inhibitor of the enzyme.

INTRODUCTION:

Pyridoxal 5'-phosphate (PLP) has been characterized as an active site directed inhibitor of D-ribulose 1,5-bisphosphate carboxylases/oxygenases (3-phospho-D-glycerate carboxylases (dimerizing), EC 4.1.1.39) by several authors (1 - 6).

PLP reacts specifically with essential amino groups at the reaction centers of these enzymes. 16 ϵ-amino groups of lysyl residues were found to be involved in the activity of the spinach carboxylase (3). PLP also inhibits the regulatory functions of these enzymes, however, presumably

Abbreviations: RuP_2 = D-ribulose 1,5-bisphosphate
PLP = pyridoxal 5'-phosphate
TNS = 2-p-toluidinonaphthaline-6-sulfonate

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there is no direct interaction between PLP and the effectors at the regulatory centers (6).

Because of its characteristic spectral properties PLP is an attractive reporter molecule for monitoring events at the reaction centers of D-ribulose 1,5-bisphosphate carboxylases/oxygenases and their vicinity. In this publication the multiple binding sites for this inhibitor have been differentiated fluorimetrically. A high affinity site of the spinach enzyme for PLP has been selectively detected and characterized.

METHODS AND MATERIALS:

Ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach was purified and assayed as described in (7). The specific activity of our preparations was 0,5 - 1,5 µmoles CO_2 fixed per min and mg protein.

Difference absorption spectra were recorded with a Perkin-Elmer two wavelength spectrophotometer model 556, equipped with a baseline correction accessory. Fluorimetric binding studies with PLP were performed with a Perkin-Elmer MPF-44 spectrofluorimeter in combination with a differential corrected spectra attachment DCSU-2. Quantum corrected difference excitation and emission spectra of enzyme-PLP-complexes were recorded with a 3 nm excitation and 10-15 nm emission band width. For each measurement the fluorescence of free PLP as a blank was subtracted from the fluorescence of the complete reaction mixture. Rhodamin B was used for the quantum correction of the fluorescence spectra. Fluorimetric studies of the fructose 1,6-bisphosphate binding using 2-p-toluidinonaphthaline-6-sulfonate (TNS) as a fluorescent reporter group were performed as described in (8).

The composition of the reaction mixtures was essentially as described in (6). Protein concentration varied between 0,1 and 0,5 mg/ml. The carboxylase was preincubated in 0,1 M bicine buffer, pH = 8,0 in the presence of either a) 10 mM $MgCl_2$; b) 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ or c) without $MgCl_2$ and bicarbonate for 20 minutes prior to the addition of PLP. For the measurement of the influence of RuP_2 and effector sugar phosphates on PLP binding the enzyme was preincubated with various concentrations of these agents in the presence of 10 mM $MgCl_2$. The reaction time of the enzyme with the inhibitor was 20 minutes. For the fluorimetric detection of the reduced form the enzyme-PLP complex was treated with 10-100 mM $NaBH_4$ for one hour. 10 ml n-octanol were added to prevent foaming of the samples.

All measurements were performed at 25°C. RuP_2, the effector sugar phosphates and PLP were products of Sigma. TNS as the potassium salt was purchased from Serva.

RESULTS AND DISCUSSION:

In Fig. 1 PLP binding to the spinach carboxylase has been studied by physico-chemical methods. Aldimine formation in the reaction between the spinach carboxylase and PLP was quantitated spectrophotometrically in Fig. 1 a.

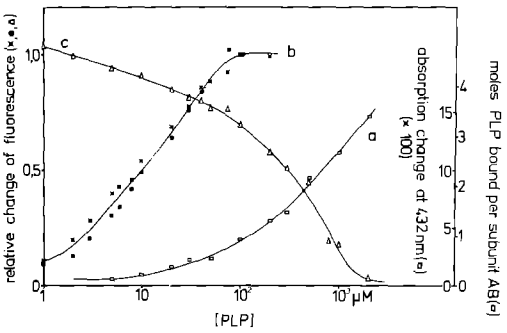

Fig. 1. Spectrophotometrical and fluorimetrical detection of PLP binding to the D-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach.
a) absorption changes at 432 nm (□);
b) fluorescence changes at 500 nm (aldimine formation; excitation wavelength: 430 nm) (●) and 390 nm (reduced complex; excitation wavelength: 325 nm) (×) and
c) fluorescence changes at 330 nm (protein fluorescence of the reduced carboxylase-PLP-complex; excitation wavelength: 260 nm) (△) as a function of the PLP concentration in the presence of 10 mM $MgCl_2$.

The absorption change at 432 nm was measured using the extinction coefficient ε = 5.800 $lmol^{-1}cm^{-1}$ as published by Paech et al. (2). In the PLP concentration range investigated (0-2 mM) up to 3 - 4 molecules of PLP per subunit AB of the carboxylase are bound. These multiple binding sites of the enzyme for the inhibitor can be differentiated fluorimetrically. PLP binding has been detected either directly by the fluorescence changes resulting from aldimine formation or its reduced derivative (Fig. 1 b) and, indirectly, by a PLP induced decrease of the intrinsic fluorescence of the carboxylase (Fig. 1 c). The PLP concentration profile of the latter effect appears biphasic resembling the spectrophotometrical titration curve. This quenching obviously originates from a perturbation of aromatic chromophores of the enzymes by PLP. An energy transfer between protein fluorophors and the bound PLP could not be observed. These experiments require high PLP concentrations up to 2 mM. In order to prevent internal absorption effects in the fluorescence measurement at high inhibitor concentrations PLP was irreversibly linked to the enzyme by reduction with $NaBH_4$ and samples of the reduced carboxylase-PLP-complexes were diluted 10 or 100 fold resp. prior to fluorescence detection.

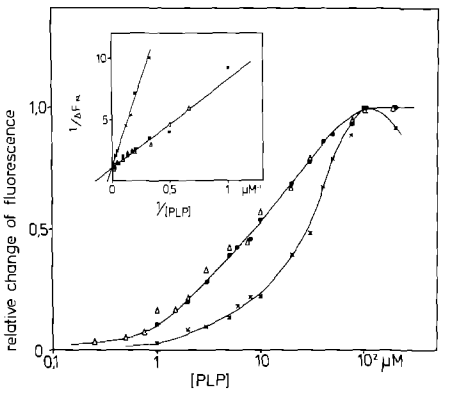

Fig. 2. Relative change of fluorescence in the formation of the reduced carboxylase-PLP-complex at 390 nm as a function of the PLP concentration. a) without Mg^{2+} and HCO_3^- (×); b) in the presence of 10 mM Mg^{2+} (●) and c) in the presence of 10 mM Mg^{2+} and 10 mM HCO_3^- (Δ). Excitation wavelength: 330 nm
Insert: Double reciprocal plots $1/\Delta F_{390}$ as a function of $1/[PLP]$.

If the fluorescence emission of the enzyme bound PLP at 500 nm (aldimine) or 390 nm (secondary amine as the reduced form) is determined as a function of the PLP concentration (Fig. 1 b) a quite different binding profile is obtained. The maximal fluorescence change levels off at 100 until 200 µM PLP. Under these conditions about 1 molecule of the inhibitor is incorporated per subunit AB of the carboxylase, as is apparent from the absorption measurements in Fig. 1 a.

PLP is more tightly bound in the presence of Mg^{2+} than without Mg^{2+}. No additional effect of $NaHCO_3$ could be observed (Fig. 2). The degree of PLP-labeling of the enzyme, however, is independent of the preincubation conditions. The Mg^{2+} induced change of the affinity of the enzyme for PLP may depend on a formation of a ternary complex of the carboxylase with the inhibitor and Mg^{2+} or, alternatively, the PLP binding sites may be modified by Mg^{2+} bound to separate sites of the enzyme. The binding profiles for PLP in Figs. 1 b and 2 a - c are consistent with a binary equilibrium reaction between the enzyme and PLP. Linear double reciprocal plots $1/\Delta F_{390}$ as a function of $1/[PLP]$ have been obtained

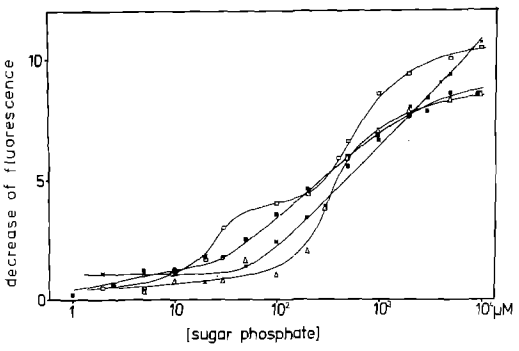

Fig. 3. The decrease of the fluorescence of the reduced carboxylase-PLP-complex at 390 nm as a function of preincubation with various concentrations of RuP_2 (△) and the effector sugar phosphates 6-phosphogluconate (●) and fructose 1,6-bisphosphate (×).
Reaction conditions: 50 µM PLP; 10 mM Mg^{2+}. Excitation wavelength: 325 nm.
For comparison the fructose 1,6-bisphosphate induced decrease of the fluorescence of the carboxylase-TNS-complex was detected (□).
Reaction conditions: 25 µM TNS; 10 mM Mg^{2+}.
Excitation wavelength: 366 nm; emission wavelength: 427 nm.

(insert in Fig. 2). From these diagrams dissociation constants for the enzyme-PLP-complex under various reaction conditions were derived. In the absence of Mg^{2+} K_D = 27 µM was found. In the presence of 10 mM Mg^{2+} alone or in combination with 10 mM HCO_3^- K_D = 7 µM was determined. Obviously PLP binding to a high affinity site of the carboxylase can be studied selectively under these experimental conditions.

The fluorescence of the enzyme-PLP-complexes is diminished, if the enzyme is preincubated with RuP_2 prior to the reaction with PLP, as is shown in Fig. 3. This result is consistent with the characterization of PLP as an active site directed inhibitor of D-ribulose 1,5-bisphosphate carboxylases/oxygenases (1 - 6). Similar conclusions can be drawn from experiments with regulatory sugar phosphates, like 6-phosphogluconate and fructose 1,6-bisphosphate. These agents bind to regulatory centers of these enzymes and enhance CO_2 fixation at low concentrations. At concentrations higher than approx. 50 mM these effectors are also attached to the reaction centers of the enzyme and inhibit the carboxylase reaction competitively to RuP_2 (7, 9).

Effector binding both to the catalytical and regulatory sites can be detected, if TNS is applied as a fluorescent marker, as is shown for fructose 1,6-bisphosphate in Fig. 3. The fluorescence of the high affinity complex of the spinach enzyme with PLP is diminished by 6-phosphogluconate and fructose 1,6-bisphosphate in the inhibition range similar to the RuP_2 effect. On the other hand PLP binding is not affected by these compounds at lower concentrations in the effector range. These findings support our previous spectrophotometrical results (6) which demonstrate that effector sugar phosphates and PLP do not interact at the regulatory centers of the enzyme. PLP, therefore, can be used as a reporter fluorophor for sugar phosphate binding specifically to the reaction centers of the carboxylase.

The results of this paper demonstrate multiple binding sites of the spinach carboxylase for PLP which can be differentiated by fluorimetric techniques. If the specific fluorescence of the enzyme-PLP-complex is monitored PLP binding to a high affinity site of the enzyme can be selectively investigated. Our observations provide additional evidence that these PLP binding sites represent the reaction centers of the carboxylase. They are in accordance with the findings of other authors (2 - 6). Paech et al. (2) found 9,5 high affinity PLP binding sites for the spinach enzyme. Whitman and Tabita (5) demonstrated that the Rhodospirillum rubrum carboxylase contains two high affinity sites for PLP and two catalytic sites per enzyme dimer. PLP binding at only one site is sufficient for the complete inactivation of this carboxylase. The difference in the kinetic patterns for PLP deactivation of those enzymes need to be clarified by additional experiments.

Our results suggest different environments of the PLP binding sites at the spinach carboxylase. PLP binding to its high affinity sites is accompanied by an increase of the quantum yield of the PLP emission com-

pared to the fluorescence of PLP in free solution. This indicates a transition to a more apolar state of the inhibitor in its enzymic environment. In contrast to this result the spectral properties of PLP remain unchanged when PLP binds to its low affinity sites of the carboxylase. These processes, however, induce a perturbation of aromatic chromophors of the enzyme probably as a consequence of PLP induced conformational changes modifying the environment of such groups.

A further characterization of the PLP binding sites of D-ribulose 1,5-bisphosphatecarboxylases/oxygenases is in preparation.

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REFERENCES:

1. Whitman, W. B. and Tabita, F. R. (1976) Biochem. Biophys. Res. Commun. 71, 1034-1039.
2. Paech, C., Ryan, R. J. and Tolbert, N. E. (1977) Arch. Biochem. Biophys. 179, 279-288.
3. Paech, C. and Tolbert, N. E. (1978) J. Biol. Chem. 253, 7864-7873.
4. Whitman, W. B. and Tabita, F. R. (1978) Biochemistry 17, 1282-1287.
5. Whitman, W. B. and Tabita, F. R. (1978) Biochemistry 17, 1288-1293.
6. Vater, J., Gaudszun, T., Scharnow, H. and Salnikow, J. (1980) Z. Naturforsch. 35 c, 416-422.
7. Vater, J. and Salnikow, J. (1979) Arch. Biochem. Biophys. 194, 190-197.
8. Vater, J., Salnikow, J. and Kleinkauf, H. (1977) Biochem. Biophys. Res. Commun. 74, 1618-1625.
9. Chu, D. K. and Bassham, J. A. (1975) Plant Physiol. 55, 720-726.