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A chromophore in glutamate decarboxylase has been wrongly identified as PQQ

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Pyrroloquinoline quinone (PQQ) has been claimed to be a component of glutamate decarboxylase from *Escherichia coli* on the basis of a frequently used procedure in which the protein is extracted with hexanol. We demonstrate that if pyridoxal phosphate (PLP) is not added during the preparation, the apoenzyme prepared from glutamate decarboxylase contains no chromophore absorbing above 280 nm. Full enzyme activity and the original holoenzyme spectrum are restored by the addition of PLP alone. A 340 nm-absorbing band, similar to that which prompted analysis for PQQ, is produced by exposure of the enzyme to solutions of PLP.

Pyrroloquinoline quinone; Pyridoxal phosphate; Glutamate decarboxylase; Cofactor; Chromophore

1. INTRODUCTION

Evidence has been presented [1] that pyrroloquinoline quinone (PQQ) is present in glutamate decarboxylase from *Escherichia coli* in an amount stoichiometric with that of pyridoxal 5′-phosphate (PLP), the cofactor that has hitherto been considered as the sole non-protein component of this enzyme [2–7]. Similar claims have been made for other amino acid decarboxylases, namely aromatic amino acid decarboxylase from pig kidney [8] and tryptophan decarboxylase from *Catharanthus roseus* [9]. A mechanism has been proposed in which both PQQ and pyridoxal phosphate act in a concerted fashion in the catalysis of amino acid decarboxylation [10].

The presence of a second cofactor in these and other enzymes, until now considered to be classically PLP-dependent, was prompted by features of their absorbance spectra, particularly by the presence of a 340 nm absorbing chromophore that is not released when the enzymes are treated by standard methods for the removal of PLP.

In the present paper we report results of experiments which demonstrate that glutamate decarboxylase from *E. coli* contains PLP as the only cofactor having absorption bands at wavelengths higher than those expected for unmodified aromatic amino acids. In addition we describe observations that reveal the origins of the 340 nm absorbing chromophore.

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2. MATERIALS AND METHODS

2.1. *Enzyme purification and assay*

Glutamate decarboxylase was prepared by the method of Yang and Metzler [11] incorporating the modifications described by Grant et al. [7] but excluding PLP from the buffer solutions used for dialysis and column chromatography where indicated. The enzyme was assayed using an unbuffered solution of glutamic acid (2 mM) containing the pH indicator Bromocresol green (0.002% w/v) that had been brought to pH 4.6 by adding KOH. The reaction was followed at 615 nm and the relationship between absorbance change and concentration change was determined by titration with a standard KOH.

2.2. *Removal of chromophore*

The preparation (5 ml, 1 mg/ml) was treated with 80 mg of α-methylglutamate so as to remove the chromophore. The method used was that of Yang and Metzler except that the solutions were maintained at 4–10°C and separations and buffer changes were achieved by forced dialysis through Centricon filters.

2.3. *Determination of absorption spectra*

Absorption spectra were determined on a Beckman Model DU 7 spectrophotometer and the data were transferred to the graphics programme Sigmaplot 4 which was used to prepare Figs 1 and 2.

3. RESULTS

Fig. 1a shows an absorption spectrum of glutamate decarboxylase prepared by the standard method in which PLP (0.1 mM) was included in the buffers used both for dialysis and chromatography. Fig. 1b shows the apoenzyme absorbance spectrum obtained from this preparation after the chromophore absorbing at 417 nm has been converted to one absorbing at 330 nm and then removed. The preparation shown in Fig. 1t had no enzyme activity.

Fig. 2a shows the absorption spectrum of glutamate decarboxylase (spec. act. 51 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at

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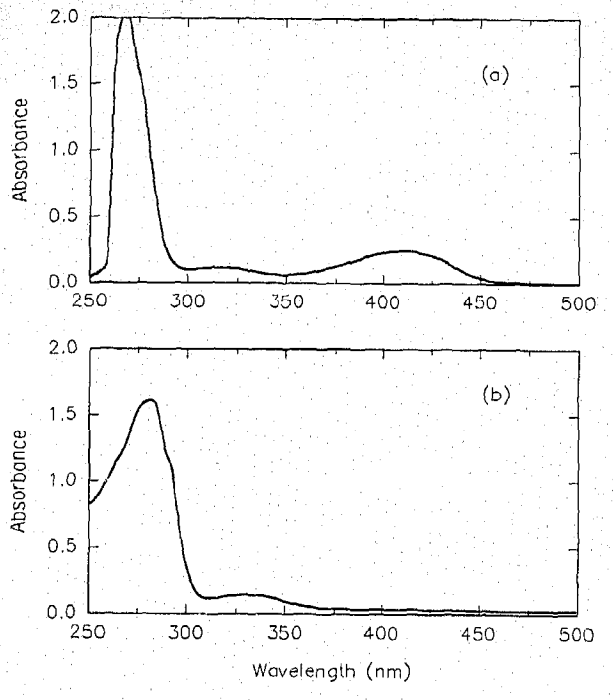

Fig. 1. Absorption spectra of glutamate decarboxylase prepared by the standard method that includes PLP in buffer solutions. (a) Before separation of cofactor. (b) After removal of all separable cofactor giving enzyme with zero activity.

25°C) prepared without adding PLP during the preparation and Fig. 2b shows the spectrum that results from treatment with α-methyl glutamate and removal of the resulting 330 nm-absorbing chromophore. The apoenzyme having the spectrum shown in Fig. 2b had no enzyme activity. Treatment with PLP to a final concentration of 6 μM, produced an enzyme with specific activity identical to that of the starting material and having the absorbance spectrum shown in Fig. 2c.

Another sample of glutamate decarboxylase, prepared without the addition of PLP and showing no absorbance at 330 nm, was maintained in the presence of 10^{-4} M PLP for 24 h exposed to the normal light of the laboratory. Excess PLP was removed from the sample by forced dialysis and the spectrum again recorded (Fig. 2d).

4. DISCUSSION

The spectrum of Fig. 2a shows clearly that only one chromophore absorbing at wavelengths above those expected for the aromatic amino acids is present in the enzyme prepared in the absence of added PLP. The absorbance maximum of this band at 417 nm is consistent with its being an imine of PLP with ε-amino group of lysine and its conversion to a 330 nm-absorbing species by treatment with α-methylglutamate confirms

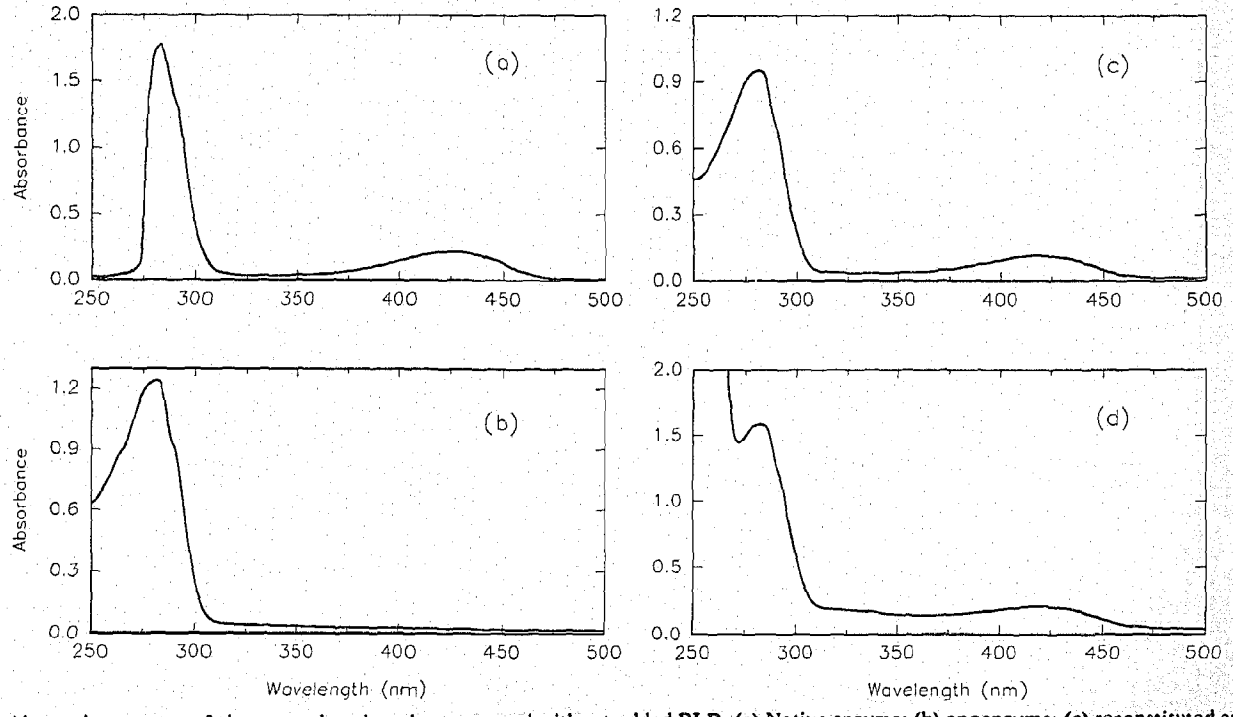

Fig. 2. Absorption spectra of glutamate decarboxylase prepared without added PLP. (a) Native enzyme; (b) apoenzyme; (c) reconstituted enzyme; (d) enzyme formed after exposure to a solution of PLP.

the previously demonstrated observations [7,12] that the enzyme has undergone a decarboxylation-dependent transamination to the pyridoxamine phosphate form. The absorbance spectrum of Fig. 2b shows that, in this form, the cofactor can be completely removed from the enzyme to leave a protein with no trace of any chromophore with an absorbance maximum above 280 nm. Bearing in mind the published spectra of free PQQ and its derivatives as well as those of established quinoproteins [13] it is inconceivable that the apoprotein having the spectrum shown in Fig. 2b could still contain PQQ. The fact that the full activity of glutamate decarboxylase, as well as the enzyme's original spectrum, are restored by the addition of PLP alone demonstrates that glutamate decarboxylase has no requirement for, and does not contain any other cofactor.

The observation that a sample of glutamate decarboxylase completely devoid of a 340 nm-absorbing band was only obtained when the enzyme was prepared in the absence of added PLP gives a strong indication that this particular chromophore arises artefactually from the interaction of the enzyme, either with the cofactor itself, or more probably with derivatives of PLP such as pyridoxic acid and 5,5'-bis(dihydroxyphosphinyloxymethyl)-3,3'-dihydroxy-2,2'-dimethyl-4,4'-pyridyl that are known to be produced in solutions of the cofactor [14]. This conclusion is confirmed by the spectrum of Fig. 2d which shows that an enzyme-associated 340 nm chromophore develops when glutamate decarboxylase is kept in a solution of PLP.

It has recently been demonstrated that two other enzymes, namely the metalloproteins, bovine dopamine hydroxylase and soy bean lipoxygenase-1 which were claimed to be quinoproteins [15,16], do not in fact contain PQQ nor any other similar cofactor [17,18]. In both these cases the method that gave a falsely positive indication of the presence of PQQ was based on extraction of the relevant enzymes after treatment with phenylhydrazine. The evidence that PQQ is a component of glutamate decarboxylase and other amino acid decarboxylases arose from treatment of the enzyme by a quite different and frequently used analytical procedure involving extraction with hexanol [19]. The fact that a falsely positive result was obtained with bacterial glutamate decarboxylase leads to the conclusion that, like the hydrazine method, the hexanol extraction procedure is an unreliable indicator that PQQ is a component of any protein. The status of the many proteins that have recently been identified as containing PQQ solely on the basis of these two methods should be reconsidered.

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