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On the biosynthesis of free and covalently bound PQQ

Glutamic acid decarboxylase from *Escherichia coli* is a pyridoxo-quinoprotein

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Analysis of glutamic acid decarboxylase (GDC) (EC 4.1.1.15) from *Escherichia coli* ATCC 11246 revealed the presence of six pyridoxal phosphates (PLPs) as well as six covalently bound pyrroloquinoline quinones (PQQs) per hexameric enzyme molecule. This is the second example of a pyridoxo-quinoprotein, suggesting that other atypical pyridoxoproteins (PLP-containing enzymes) have similar cofactor composition. Since the organism did not produce free PQQ and its quinoprotein glucose dehydrogenase was present in the apo form, free PQQ is not used in the assemblage of GDC. Most probably, biosynthesis of covalently bound cofactor occurs in situ via a route which is different from that of free PQQ. Thus, organisms previously believed to be unable to synthesize (free) PQQ could in fact be able to produce quinoproteins with covalently bound cofactor. Implications for the role of PQQ in eukaryotic cells are discussed.

Pyrroloquinoline quinone; Cofactor; Quinoprotein; Glutamic acid decarboxylase; Glucose dehydrogenase; Pyridoxal phosphate; (*E. coli*)

1. INTRODUCTION

A large number of Gram-negative bacteria produce PQQ in their mineral culture medium when grown on substrates like methanol, ethanol or quinic acid, substrates which are frequently converted via a quinoprotein dehydrogenase by these organisms [1]. Those quinoprotein dehydrogenases investigated with respect to their localization appear to occur in the periplasm. Therefore, the presence of PQQ in the spent medium can be explained by assuming that the protein is transported in unfolded form across the cytoplasmic membrane and that reconstitution to holoenzyme occurs in the periplasm with PQQ which can escape through the outer membrane to the medium. The fact that immediate reconstitution to holoenzyme is observed on addition of PQQ to cells of PQQ^- mutants is in accordance with this view. Perhaps this phenomenon has significance in the natural environment in view of the occurrence of bacteria with quinoprotein dehydrogenase apo-enzymes and of PQQ overproducers [2].

Thus far quinoprotein dehydrogenases have not been detected in eukaryotic cells. On the other hand, several enzymes with covalently bound PQQ have been observed in eukaryotes [3]. Since many occur in mammals, the question as to whether PQQ functions as a vitamin is relevant. However, nothing is known about mammalian PQQ biosynthesis or the manner of assemblage of these quinoproteins so that the question cannot be answered at the moment.

Tyrosine and glutamic acid are the precursors of bacterial PQQ biosynthesis [4,5]. In a search for intermediates in the route of biosynthesis, using 4 genetically different classes of PQQ^- mutants, no cross-feeding was observed [6]. Therefore, it has been suggested that the whole process occurs on a matrix where ring closure, hydroxylation, and ox-

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idation proceed without any free intermediates, the final step being the excision of PQQ [7]. Extending this view, synthesis of the covalently bound cofactor does not require free PQQ but occurs in situ, i.e. in the quinoprotein itself, thus explaining why free PQQ has not been detected so far in mammals. The latter observation could, however, also be explained in a different way, namely by assuming that the cofactor is produced but cannot be detected due to the complexity of mammalian systems. For instance, it is known that free PQQ readily reacts with nucleophilic compounds (e.g. amino acids) and the products formed are undetectable in a bioassay using a quinoprotein dehydrogenase apo-enzyme [3,7]. Therefore, to verify the hypothesis it would be necessary to examine an organism where the absence of free PQQ can be reliably assessed and subsequently to determine whether it produces quinoproteins where the cofactor is covalently bound.

Those common *Escherichia coli* laboratory strains which have been investigated appear to contain quinoprotein glucose dehydrogenase in the apo-form [8], indicating the absence of free PQQ. It is also known that certain strains produce glutamic acid decarboxylase (GDC) (EC 4.1.1.15), an enzyme which requires pyridoxal phosphate (PLP) for activity [9]. Based on its spectral behaviour when adding substrate, substrate analogues [10] or the inhibitor hydroxylamine [11], GDC is an atypical pyridoxoprotein (PLP-dependent enzyme). Since we recently found that a representative of this group, namely pig kidney aromatic-L-amino acid decarboxylase (EC 4.1.1.28), contains covalently bound PQQ [12], GDC was investigated in order to ascertain the ability of *E. coli* to produce this form of the cofactor.

2. MATERIALS AND METHODS

2.1. *Purification of GDC*

GDC from *E. coli* ATCC 11246 (Sigma, type V) was purified by FPLC on a Superose HR 12 gel-filtration column (Pharmacia) in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Fractions having an absorbance ratio of $A_{280nm}/A_{340nm} = 12$ (a ratio which can be derived from the absorption spectrum presented in [13]) were pooled. This preparation was adequate for cofactor analysis, since its absorption spectrum was identical to that reported for a homogeneous preparation [13]. Enzyme concentrations were calculated from the absorbance at 280 nm, measured in 0.1 M potassium phosphate buffer (pH 7.0), using an $A^{1\%}_{280nm}$ value of 17 (at pH 7.0) and an M_r of 310000 [14].

2.2. *PLP analysis*

The PLP content of the enzyme preparation was determined as described by Wada and Snell [15] as well as via the alkaline method [11,16] measuring the absorbance at 388 nm in 0.1 M NaOH and using a molar absorption coefficient of 6600 $M^{-1} \cdot cm^{-1}$.

2.3. *PQQ analysis*

Analysis was performed using the previously described [12] hexanol extraction procedure, except that some modifications were incorporated leading to only a single product. To a solution of 7.5 ml enzyme (0.116 mg/ml) in 30 mM potassium phosphate buffer (pH 6.3) containing 30 mM NaCl, 7.5 ml of 6 M HCl and 10 ml hexanol were added. The mixture was refluxed for 30 min, after which the condenser was removed so that the water could evaporate. The remaining hexanol phase was refluxed for a further hour, transferred to a film evaporator and the hexanol removed under reduced pressure. The residue was dissolved in 1 ml methanol. HPLC was performed on a C_8 reversed-phase column (Merck LichroCart 125 × 4 mm; 4 μm particle size), using methanol with 0.5% H_3PO_4 as eluant at a flow rate of 1.5 ml/min. Detection occurred with a Hewlett-Packard HP1040 A photodiode array detector, monitoring the eluting peaks at 318 nm and recording spectra throughout the chromatogram.

2.4. *Determination of free PQQ*

Lyophilized *E. coli* ATCC 11246 cells (Sigma) were extracted with 90% methanol, acidified to pH 2.0 with a HCl solution. After neutralization, free PQQ was determined in a biological assay [17]. The amount of quinoprotein glucose dehydrogenase holo-enzyme activity was determined as in [18]. Apo-enzyme 'activity' was determined by incubating cell-free extract with 0.1 mM PQQ.

3. RESULTS

3.1. *PLP and PQQ in GDC*

Based on the procedure of Wada and Snell [15] and the alkaline method [11,16], the GDC preparation contained 5.1 and 7.4 PLP molecules per enzyme molecule, respectively. In view of the relatively mild extraction conditions in the first method, PLP is non-covalently bound.

HPLC of the residue obtained with the hexanol extraction method gave a product with retention time and absorption spectrum identical to those of the product prepared from authentic PQQ under the same conditions (fig.1). The slightly different conditions, as compared with those in [12], led to only a single product. ^{1}H-NMR revealed it to be 4-hydroxy-4-hexoxy-PQQ, having a molar absorption coefficient of 39900 $M^{-1} \cdot cm^{-1}$ at 318 nm (to

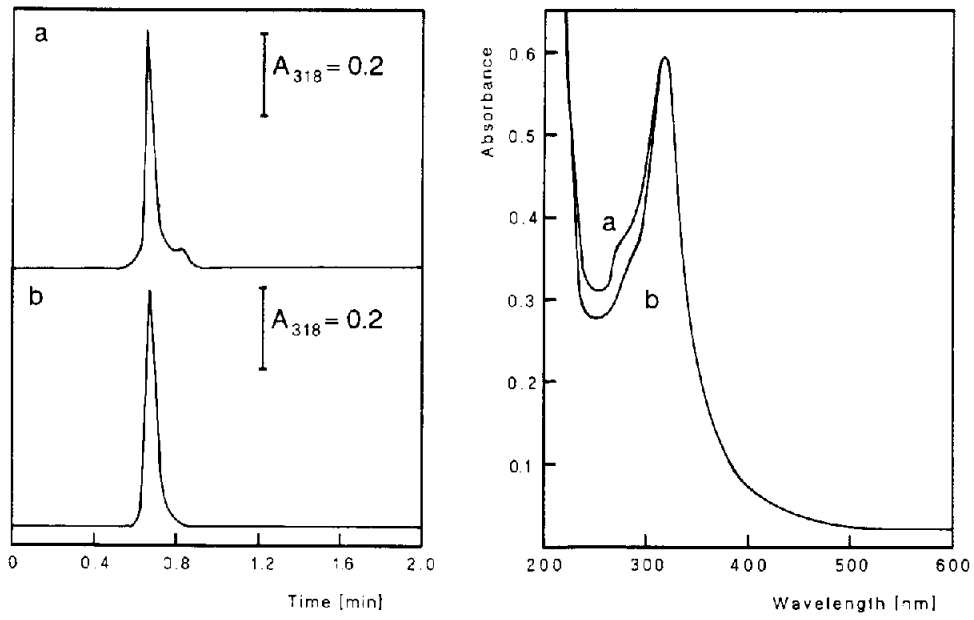

Fig.1. Chromatograms (left) and absorption spectra (right) of products obtained with the hexanol extraction procedure: (a) product extracted from GDC; (b) product prepared from authentic PQQ (4-hydroxy-5-hexoxy-PQQ). HPLC and photodiode array detection were performed as described in the text.

be published elsewhere). Using this value, it was calculated that the GDC preparation contained 5.3 PQQ molecules per enzyme molecule [no free PQQ was detected after acid-methanol extraction (see section 3.2) so that it is covalently bound]. Since GDC is a hexameric enzyme [14], this could indicate that each subunit contains one PLP and one covalently bound PQQ.

3.2. *Production of free PQQ*

Free PQQ was not detected in the extract from the cells. The cells contained glucose dehydrogenase apo-enzyme (10 nmol Wurster's Blue reduced/min per mg protein) but not holo-enzyme activity.

4. DISCUSSION

GDC is the second amino acid decarboxylase where the presence of covalently bound PQQ has been established, suggesting that other atypical pyridoxoproteins (present in the group of decarboxylase as well as transaminases [12]) might also be pyridoxo-quinoproteins. A tentative proposal for a mechanism has already been put forward for dopa decarboxylase [3]. In the reaction cycle, PQQ is active in the decarboxylation step and PLP functions as an acceptor for the amine product after which hydrolysis occurs. Since data suitable for deriving such a mechanism for GDC are rather sparse, a clear role of PQQ cannot be indicated. Nevertheless, it is very likely that the current mechanism for GDC, which is at present based solely on PLP as cofactor, should be revised in the near future.

The finding of PQQ in GDC, but not in glucose dehydrogenase or in the cell (in free form), can be explained in several ways:

(i) *E. coli* produces free PQQ at a level adequate for assemblage of GDC but not for glucose dehydrogenase.

(ii) The route of PQQ biosynthesis in *E. coli* is incomplete and the intermediate which is formed is suitable for GDC but not for glucose dehydrogenase formation.

(iii) Biosynthesis of the covalently bound cofactor does not start with free PQQ but proceeds in situ, i.e. in the quinoprotein itself.

Explanation (i) is unlikely, since very low levels of PQQ are detectable with the assay used [18]. Ex-

planation (ii) is also unlikely, since it is expectable that such a compound would have been detected in the search for (free) intermediates [6]. Therefore, explanation (iii) seems the most attractive at the moment. It suggests that biosynthesis of free and covalently bound cofactor proceeds via different routes. This is in accordance with the observation that production of glucose dehydrogenase holoenzyme in *E. coli* requires incorporation of all 4 genes for (free) PQQ biosynthesis cloned from *Acinetobacter calcoaceticus* LMD 79.41 [19].

Further support for explanation (iii) is provided by recently obtained insights into the structure of methylamine dehydrogenase (EC 1.4.99.3). This bacterial quinoprotein contains covalently bound PQQ (observed as the PQQ-hydrazone) when the hydrazine method is applied to it [20]. However, the determination of the 3-dimensional structure suggests that the cofactor in the active site is pro-PQQ, having the quinone-indole structure of PQQ connected with a glutamic acid residue which is bound to the protein chain at 2 positions (Vellieux, F.M.D. et al., to be published elsewhere). Obviously, ring closure of the glutamic acid moiety occurs easily during application of the hydrazine method (closure of this ring also proceeds smoothly in one of the chemical synthesis routes of PQQ [21]). Therefore, all other quinoproteins with covalently bound PQQ (established with the hydrazine method, and perhaps also with the hexanol extraction procedure) might in fact contain pro-PQQ, thus representing another argument in favour of the presumption that cofactor biosynthesis occurs in situ in these cases.

In summary, the detection of a quinoprotein in *E. coli* suggests that organisms previously believed to be unable to produce PQQ are in fact capable of producing covalently bound cofactor (although it remains to be established whether it is in the form of PQQ or pro-PQQ). Since most of the enzymes with covalently bound PQQ play a crucial role in the cell [7], it seems that quinoproteins are omnipresent although the ability to produce free PQQ is restricted to certain Gram-negative bacteria. This view implies that cofactor biosynthesis occurs in situ, excluding a direct effect as vitamin of free PQQ in eukaryotic cells.

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