CELL-FREE SYNTHESIS OF 6-HYDROXY-D-NICOTINE OXIDASE CONTAINING COVALENTLY BOUND FAD

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1. Introduction

6-Hydroxy-D-nicotine oxidase (6-HDNO) (EC 1.5.3.6) is an inducible flavoprotein involved in the degradation of D-nicotine by *Arthrobacter oxidans* [1,2]. The enzyme contains FAD bound covalently via a linkage between the 8α-methyl group of the flavin nucleus and N(3) of a histidyl residue of the apoprotein [3]. In recent years, several enzymes were reported to contain FAD covalently bound to the apoprotein (reviewed [4]). However, the reaction mechanisms by which the flavin cofactor is attached to the protein are still unknown.

Studies on the covalent binding of FAD in vivo led to the conclusion that, in D-nicotine-induced wild-type cells, the synthesis of the enzyme-specific mRNA limits the rate of holoenzyme synthesis [5]. However, a riboflavin-requiring mutant was obtained in which the formation of active 6-HDNO could be made flavin-dependent by partial riboflavin starvation. With the help of antibiotics acting on different steps of protein biosynthesis, the flavinylation was shown to depend on the concomitant operation of the translation process [6]. In order to accomplish the translation and flavin attachment required for 6-HDNO synthesis in a cell-free system, polysomes isolated from induced cells and an *E. coli* MRE 600 supernatant fraction were used.

Abbreviations: 6-HDNO, 6-hydroxy-D-nicotine oxidase; PAGE, polyacrylamide disc-gel electrophoresis; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid

2. Materials and methods

The riboflavin-requiring mutant of *A. oxidans* used in this study is described [5]. Polysomes were obtained as reported [7]. *E. coli* MRE 600 extracts required for the translation assay were prepared from cells of the logarithmic phase by the method in [8]. The reaction mixture for cell-free protein synthesis (total vol. 100 μl) contained 65 mM Tris–HCl buffer, pH 7.8, 82 mM NH_4Cl, 16 mM Mg-acetate, 8 mM mercaptoethanol, 3.3 mM ATP, 0.4 mM GTP, 10 mM phosphoenolpyruvate, 0.05 mM each of the unlabelled amino acids, 30 μM [^{3}H]leucine (spec. radioact. 12.3 Ci . mmol^{-1}), 4 μg pyruvate kinase (EC 2.7.1.40) and 5 μl *E. coli* S-100 or S-30 extract. This mixture was preincubated for 5 min at 37°C, 20 μl polysomes (4–8 A_{260} units . ml^{-1}) were then added and translation allowed to proceed for 15 min. SDS–PAGE was performed as in [9] with slight modifications [10]. The protein of the reaction mixture was precipitated by adding 1 ml 12.5% TCA and the precipitate washed with 1 ml TCA and 1 ml acetone. The dried pellet was dissolved in 0.1 ml SDS–sample buffer [10] and heated for 7 min at 96°C. The dissolved proteins were separated on 10% polyacrylamide gels containing 0.1% SDS. For immunoprecipitation, the total reaction mixture (100 μl) and 100 μl antiserum against 6-HDNO [11] were incubated in the presence of a final concentration of 0.4% NaCl together with 50 μl of an *A. oxidans* cell extract containing 70 mU of 6-HDNO. About 99% added carrier enzyme activity was precipitated after incubation at 37°C overnight. The precipitate was washed twice with 0.9% NaCl solution and dissolved in 100 μl SDS–sample buffer.

3. Results and discussion

The translation system used was obtained from a ribonuclease-free strain of *E. coli* (MRE 600) (table 1). Polysomes were isolated both from induced and uninduced *A. oxidans* cells. The activity of 6-HDNO begins to appear after *A. oxidans* has reached the stationary phase of growth [2], and therefore it would be expected that only a limited number of proteins are synthesized during that period.

The analysis of the translation products by SDS–PAGE (fig.1) showed one major peak indicating the preferential formation of one protein. The labelling of this protein was found to depend on the amount of polysomes added. Based on the position of marker proteins, the labelled protein had approx. mol. wt 50 000 which is the same as that of purified 6-HDNO [13]. Identical migration in SDS–PAGE was confirmed more directly by electrophoresis of a mixture of purified [^{14}C]flavin-labelled 6-HDNO and translation products (fig.1). No translation product of a similar molecular weight was detectable in a control experiment performed under identical conditions with polysomes isolated from uninduced cells (fig.1). The addition of flavins had a marked influence on the formation of the 6-HDNO-like protein although it did not alter significantly the total incorporation of [^{3}H]leucine into TCA-precipitable material (table 1). These experiments also indicate that the enzyme system necessary for the covalent binding of flavin was present in the in vitro system used. The nature of the precursor of covalently bound flavin cannot be

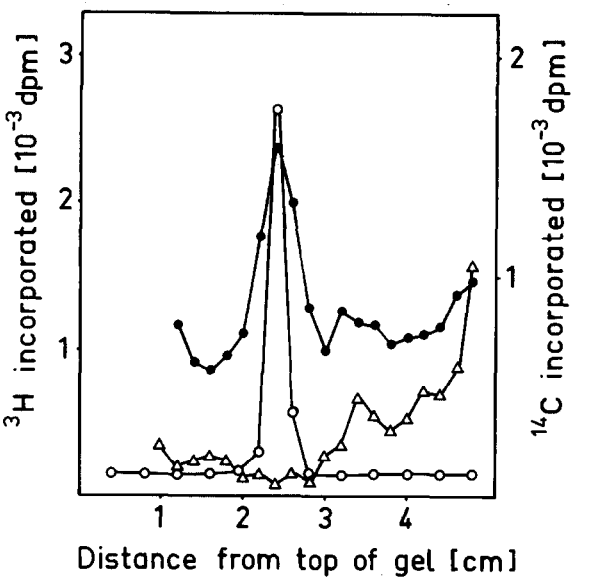

Fig.1. Separation of the products of cell-free translation. After SDS–PAGE, 2 mm slices were eluted with Tris–HCl buffer, pH 7.5, containing 10 mM mercaptoethanol, for about 7 h. Radioactivity of the eluates was determined by liquid scintillation spectrometry. 0.7 A_{260}-units of polysomes from induced (•–•) or from uninduced (△–△) cells were added. Purified [^{14}C]riboflavin-labelled 6-HDNO (○–○) was added to an unlabelled translation assay.

Table 1
[^{3}H]Leucine incorporation into TCA-precipitable material by the cell-free translation system

Additions to the reaction mixture	Incorporation rate (dpm.min^{-1})
5 μl S 100 (*E. coli*)	< 30
20 μl polysomes	< 30
20 μl polysomes + 2 μl S 100	3180
20 μl polysomes + 5 μl S 100	3990
20 μl polysomes + 10 μl S 100	2250
20 μl polysomes + 5 μl S 100 + 25 μM riboflavin + 25 μM FAD	3660

The reaction mixture is described in section 2. Polysomes were from induced cells. Samples, 10 μl, were taken from the reaction mixture at 5 min intervals; the reaction was linear with time for 20 min. Protein-bound radioactivity was determined as in [12]

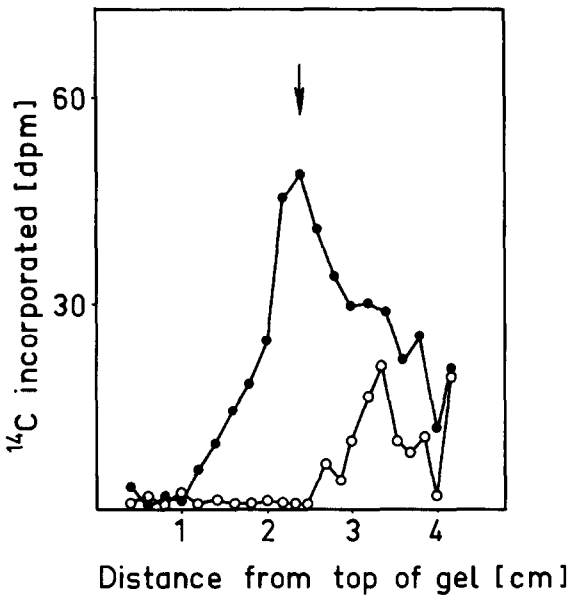

Fig.2. Covalent incorporation of flavin into 6-HDNO during cell-free translation. Standard translation assays (18) were incubated in the presence of 0.1 μCi [^{14}C]riboflavin with 0.7 A_{260}-units of polysomes from induced (•–•) and non-induced (○–○) cells, respectively. After addition of 200 mU of unlabelled 6-HDNO, each set was pooled and subjected to PAGE at pH 9 as in [13]. The eluted fractions containing enzyme activity were concentrated and analyzed by SDS–PAGE as in fig.1.

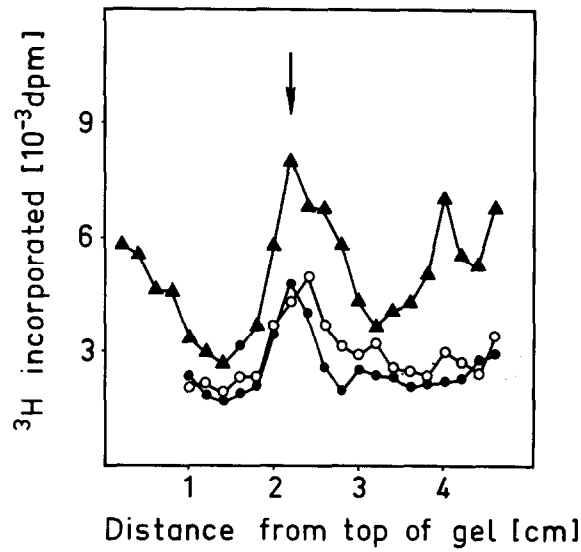

Fig.3. SDS–PAGE of immunoprecipitate (▲–▲) and of proteins synthesized in the presence (•–•) and absence (○–○) of FAD and riboflavin. Experimental procedures as in fig.1. Position of authentic 6-HDNO marked by arrow.

deduced from these experiments. However, it is probably FAD since this compound is incorporated as well as riboflavin and the translation system used was found to convert riboflavin to FAD (data not shown). The identification of the major protein formed in the cell-free system as 6-HDNO was further substantiated in translation experiments using ^{14}C-labelled riboflavin and polysomes from induced cells. It was found that ^{14}C was bound covalently to the protein which migrated identically in SDS–PAGE with 6-HDNO (fig.2). There was no incorporation of labelled riboflavin into any protein when polysomes from uninduced cells were used. Since it was found [6] that 6-HDNO is the only protein with covalently bound flavin synthesized after induction with D-nicotine, the above results provide convincing evidence for the identity of the product of cell-free translation with 6-HDNO.

Omission of flavin cofactors in the cell-free translation system containing polysomes from induced *A. oxidans* cells resulted in the formation of a protein whose position in SDS–PAGE differed consistently (10 independent analyses) by 2–3 mm from that obtained in the presence of flavins (fig.3). It appears that under these conditions a flavin-free protein accumulated which may be the apoform of 6-HDNO. The molecular weight of FAD could account for the different migration in SDS–PAGE.

The proteins synthesized in vitro by polysomes from induced cells in the presence of flavins were further analyzed by immunoprecipitation followed by SDS–PAGE of the dissociated immunoprecipitate (fig.3). The ^{3}H-labelled translation product was precipitated by an antiserum against 6-HDNO and migrated as a single peak having the same velocity as purified ^{14}C-labelled 6-HDNO run simultaneously in a control gel.

The combination of an *E. coli* (MRE 600) extract and polysomes from induced *A. oxidans* cells has permitted for the first time the cell-free synthesis of a flavoprotein with FAD attached covalently.

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