RESEARCH COMMUNICATION

Formation *in vitro* of the 3,4,6-trihydroxyphenylalanine quinone cofactor

Steven P. HANLON, Karen CARPENTER, Abby HASSAN and Ronald A. COOPER*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

An *Escherichia coli* K-12 2-phenylethylamine oxidase gene with a mutated leader sequence region produced a largely inactive form of the enzyme in the cytoplasm. This form of the enzyme was activated 30–50-fold on incubation at 30 °C in the absence

of any added cofactors. After activation the enzyme contained a quinone which was not detected in the non-activated form. This is the first report of the formation *in vitro* of any quinoenzyme cofactor.

INTRODUCTION

A novel quinone, pyrroloquinoline quinone (PQQ), was first identified 15 years ago as the dissociable cofactor of methanol dehydrogenase [1,2], and subsequently was found as the cofactor of a large number of other oxidizing enzymes [3]. In the last few years two other quinone redox cofactors, 3,4,6-trihydroxyphenylalanine quinone (TPQ) as the organic cofactor of the copper-containing amine oxidases [4], and tryptophan-tryptophyl quinone (TTQ) as the cofactor of methylamine dehydrogenase [5], have been discovered. In these latter two cases the quinones are part of the polypeptide chain and consequently non-dissociable. Enzymes that require these quinone cofactors have been called, collectively, quinoenzymes, and the topic has been covered very recently in a comprehensive book and a review [6,7].

A number of intriguing aspects of quinoenzymes remain to be resolved, and prominent among these is the mechanism of biogenesis of the quinone cofactors themselves. Despite considerable effort, the precise mode of PQQ formation is not yet known, although it is clear that the products of several genes are required. It has been reported that the biogenesis of TTQ involves the products of a number of genes, but again the process of formation is unknown. No accounts of TPQ biogenesis *in vitro* have been reported, but two possible post-translational mechanisms for its formation have been proposed. In one, separate oxidative enzyme(s) convert a polypeptide-chain tyrosine residue into TPQ, whereas the alternative proposal involves an autocatalytic 'self-processing' event in which the copper would play a key role in modification of the tyrosine residue [8].

In this communication we present details of the cell-free biogenesis of the TPQ cofactor of an *Escherichia coli* K-12 amine oxidase. The process is consistent with an autocatalytic event, and is the first-ever report of the cell-free biogenesis of any quinoenzyme cofactor.

MATERIALS AND METHODS

Growth and fractionation of cells

Cells of *E. coli* K-12 strain JM109 carrying plasmids pKC13 and pAH15 were grown on M63 minimal medium supplemented with succinate (15 mM) as carbon source and with ampicillin at 100 µg/ml. When induction of the plasmid-encoded amine oxidase gene was required, isopropyl β-D-thiogalactopyranoside

(IPTG) was added to a concentration of 0.5 mM. Cells were harvested in the mid-to-late-exponential phase of growth at an A_{680} of 0.5–0.8. Periplasmic and spheroplast fractions were prepared by using the method described previously [9]. Normal crude cell extracts (2–4 mg of protein/ml) were prepared as described previously [10]. For the preparation of concentrated crude cell extracts (15–25 mg of protein/ml) from *E. coli* JM109 [pAH15], 2 litres of cells were grown as described above, harvested, and the pellet was resuspended in 10 ml of 0.1 M sodium phosphate buffer, pH 6.0. The cell suspension was sonicated at 0 °C for 30 s at an amplitude of 10 µm, using an MSE Soniprep 150 ultrasonicator. Unbroken cells were pelleted by centrifugation at 20000 g for 20 min at 4 °C. The low-speed supernatant was then ultracentrifuged at 170000 g for 2 h at 4 °C to remove cytoplasmic membranes. The protein content of the extracts was measured by using the Folin reagent, with BSA as standard [11].

Enzyme assay

Amine oxidase was assayed with 2-phenylethylamine as substrate by the continuous spectrophotometric method [10]. When inhibition of amine oxidase activity by semicarbazide was investigated, the enzyme sample to be assayed was incubated in the cuvette which contained assay buffer (0.1 M sodium phosphate, pH 6.0) and semicarbazide (2 mM). After 5 min the assay was started by addition of 2-phenylethylamine to 1 mM.

Activation of amine oxidase in JM109 [pAH15] extracts

JM109 [pAH15] cell extract (15–25 mg of protein/ml; 1 ml) was diluted into 100 ml of 0.1 M sodium phosphate buffer, pH 6.0, and maintained at 30 °C. A further sample was diluted likewise and maintained at 0 °C. When required, EDTA (pH 6.0) was added to a final concentration of 0.1 mM. Samples (1 ml) were removed immediately from all three incubations, and then at 10 min intervals, for a period of 3–4 h, for assay of amine oxidase activity. When maximal activity had been reached, the remaining diluted extracts (approx. 80 ml) were stored on ice before concentration by ultrafiltration at 4 °C through an Amicon YM 10 (molecular-mass cut-off 10 kDa) membrane. The concentrated samples (approx. 10 ml) were freeze-dried, and the residue was dissolved in 0.5 ml of distilled water.

Abbreviations used: PQQ, pyrroloquinoline quinone; TPQ, 3,4,6-trihydroxyphenylalanine quinone; TTQ, tryptophan-tryptophylquinone; IPTG, isopropyl β-D-thiogalactopyranoside; PAC, phenylacetic acid.

* To whom correspondence should be addressed.

Detection of quinone

Samples were subjected to SDS/PAGE [12], except that 2-mercaptoethanol was omitted from the sample buffer. Proteins were electroblotted on to a nitrocellulose membrane by using an LKB semi-dry blotting apparatus and analysed for quinone by the Nitro Blue Tetrazolium/glycinate redox cycling method [13].

Nucleotide sequencing

Nucleotide sequencing was carried out with T7 DNA polymerase [14]. Plasmid DNA for sequencing was isolated as described [15].

PCR procedures

Primers used for amplifying the normal *maoA* gene were (a) 5'-CTGAGAATTCGCCACAGAGCGCGG3' and (b) 5'-CAG-GGGAATTCGTGATTTGGCCCCG3'. The mutated form of the *maoA* gene was prepared by using primer (b) above with primer (c), 5'-GCCTGGAATTCGAGGTTATTTATGCACGG-TGGT3'. The amplification reactions contained, in a final volume of 50 μ l: 100 ng of template DNA (pJPB13), 25 pmol each of primers a and b or b and c, 250 pmol of each dNTP, in 20 mM Tris/HCl, pH 8.8, containing 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM MgSO_4 and 0.1% Triton X-100. After an initial denaturation at 95 °C for 5 min, followed by cooling, 1 unit of Vent polymerase (New England Bio-Labs) was added. A total of 25 cycles of the following parameters were used: denaturation, 95 °C for 3 min; annealing, 64 °C for 1.5 min; extension, 72 °C for 3 min. The DNA product was isolated, after electrophoresis on 0.8%-agarose gels, by using a silica matrix (GeneClean II; Bio 101 Inc., La Jolla, CA, U.S.A.), for cloning into pUC18 by using a Sureclone kit and the related protocol (Pharmacia).

RESULTS AND DISCUSSION

Cloning normal and leader-sequence-mutated forms of the *E. coli* K-12 amine oxidase structural gene

Phenylacetic acid (PAC) is an intermediate in the utilization of 2-phenylethylamine by *E. coli* K-12 [10]. Various genes for PAC catabolism are located on plasmids pJPB13 and pBS1 [16], and we found that the gene for 2-phenylethylamine oxidase (amine oxidase) was also on pJPB13 (S. Parrott and R. A. Cooper, unpublished work).

We noted that the N-terminal amino acid sequence of a purified *Klebsiella aerogenes* tyramine oxidase encoded by the *maoA* gene [17] was virtually identical with the N-terminal amino acid sequence of purified *E. coli* K-12 amine oxidase (A. Hacisalihoglu and R. A. Cooper, unpublished work). This suggested that these two amine oxidases were, in fact, essentially identical enzymes, so to obtain nucleotide sequence information on the *E. coli* K-12 *maoA* gene we used the *K. aerogenes* *maoA* deduced amino acid sequence to construct redundant oligonucleotides for use as sequencing primers [18]. This allowed us to obtain some nucleotide sequence upstream of the 5' end, and downstream of the 3' end, of the *E. coli* K-12 *maoA* gene (results not shown). This sequence information was used to construct primers for use in PCR procedures specifically to clone only the *maoA* structural gene without the flanking DNA. This *E. coli* K-12 *maoA* gene sequence was placed under the control of the pUC18 *lac* promoter to give plasmid pKC13. In addition, we constructed, by PCR procedures, a form of the *E. coli* K-12 *maoA* gene in which the normal 30-amino-acid signal sequence (MGSPSLYSARKTTTLALAVALSFAWQAPVFA) [19] was replaced by a different 21-amino-acid signal sequence

(MTMITNSSSSVPAWNSRLFM) lacking the residue acted on by the signal peptidase. This latter construct, again under the control of the pUC18 *lac* promoter, was designated pAH15. These two forms of the amine oxidase gene have been used to investigate the biogenesis of the TPQ cofactor.

Expression of amine oxidase by JM109 [pKC13]

pKC13 was used to see if it was possible to identify growth conditions under which the amine oxidase protein was made in a form lacking the quinone cofactor. When JM109 [pKC13] was grown on succinate minimal medium in the presence of ampicillin, the activity of amine oxidase in cell-free extracts was barely detectable (0.005 μ mol/min per mg of protein), but, when 0.5 mM IPTG was included, a very high amine oxidase activity was observed (1.5 μ mol/min per mg of protein) and the corresponding strong 80 kDa protein band was seen by SDS/PAGE. Fractionation of succinate/ampicillin/IPTG-grown cells into periplasm and spheroplasts showed that the amine oxidase produced from the cloned gene was present exclusively in the periplasm, as is the case for the enzyme produced by the original organism, *E. coli* K-12.

Growth of JM109 [pKC13] on Luria broth/ampicillin/IPTG resulted in a lower specific activity of amine oxidase in crude extracts (0.11 μ mol/min per mg of protein), as did growth on glucose/ampicillin/IPTG (0.46 μ mol/min per mg of protein). Growth on galactose/ampicillin/IPTG resulted in high specific activity of the amine oxidase (1.7 μ mol/min per mg of protein). Propagation of pKC13 in other *E. coli* strains growing on galactose/ampicillin/IPTG resulted in a range of amine oxidase specific activities: in *E. coli* C 0.54 μ mol/min per mg of protein, in *E. coli* B 0.4 μ mol/min per mg of protein, and in *E. coli* W 0.7 μ mol/min per mg of protein. When, from such crude extracts, an equal number of amine oxidase activity units were analysed by SDS/PAGE, the intensity of the 80 kDa amine oxidase protein band was constant, indicating that none of the growth conditions led to the formation of significant amounts of catalytically inactive 80 kDa protein. It seemed that variation in the measured specific activity of the amine oxidase produced from pKC13 reflected directly the amount of fully active enzyme present.

If the products from other genes were needed for quinone cofactor formation, they would not be encoded on the pKC13 DNA or induced by IPTG, but obviously such genes, if they exist, could be present in the host organism in which the cloned amine oxidase gene is expressed. Growth of JM109 [pKC13] on succinate/ampicillin/IPTG led to 10 times more amine oxidase activity than the organism itself produced optimally during growth on 2-phenylethylamine, and 1000 times more than it produced during growth on succinate [10]. If the host organism was producing modifying enzyme(s) needed to form the quinone cofactor, such enzyme(s) must therefore be produced constitutively and be non-rate-limiting for quinone formation. An alternative explanation for the ready formation of the active amine oxidase was that the *maoA* gene product was able to form its quinone cofactor autocatalytically.

Expression of amine oxidase by JM109 [pAH15]

It was anticipated that the amine oxidase produced from pAH15 encoding the amine oxidase structural gene with its altered leader sequence would be located in the cytoplasm rather than in the periplasm, and that this changed location might affect the enzyme's activity. A protein of 80 kDa was seen by SDS/PAGE of crude extracts from JM109 [pAH15] grown on succinate/

Figure 1 Effect of dilution on activation of amine oxidase

Samples of a crude extract prepared from JM109 [pAH15] (protein = 16.0 mg/ml) were diluted into 0.1 M sodium phosphate buffer, pH 6.0, and incubated at 30 °C for 150 min.

ampicillin medium with IPTG, and this band was absent from extracts of cells grown in the absence of IPTG. When JM109 [pAH15] cells grown on succinate/ampicillin/IPTG were fractionated into periplasm and spheroplasts, the 80 kDa protein was not present in the periplasm, but was detected on ultrasonication of the spheroplast fraction. Although the growth of JM109 [pAH15] in the presence of IPTG, and the extract preparation, were exactly as for JM109 [pKC13], the amount of the 80 kDa protein seen on the gels was about 10 % of that seen for JM109 [pKC13]. However, the specific activity of the amine oxidase produced by JM109 [pAH15] ($0.005 \mu\text{mol/min per mg}$ of protein) was only 0.3 % of the value for JM109 [pKC13], suggesting that in this case an inactive form of the amine oxidase was being produced. Extracts from cells of JM109 [pAH15] grown in the absence of IPTG had no detectable amine oxidase activity.

Activation of amine oxidase

Although the crude extract from JM109 [pAH15] showed very low initial rates in the amine oxidase spectrophotometric assay, the rate increased significantly if the reaction was allowed to run for 30–60 min at 30 °C. To see if this activation was due to hydroxylation leading to TPQ formation, samples of crude extract supplemented with ATP, NADH or NADPH, the co-factors normally required by hydroxylating enzymes, were incubated for 60 min at 30 °C, but this gave little or no increase in their amine oxidase activity. A similar negative outcome was observed when a sample of extract was incubated with 1 mM 2-phenylethylamine at 30 °C for 60 min.

To find out whether the failure to achieve an increase in amine oxidase activity in the undiluted crude extract was due to inhibition of enzyme activity, various amounts of JM109 [pAH15] extract were mixed with extract from JM109 [pKC13]. The resultant activity was exactly that expected from the respective activities of the two extracts, suggesting that there was no inhibition of enzyme activity by the JM109 [pAH15] extract.

To investigate the activation further, the normal crude extract (2–4 mg of protein/ml) was incubated at 30 °C after 20-fold dilution in 0.1M sodium phosphate buffer, pH 6.0, in the absence of any added compounds, and a time-dependent increase in amine oxidase activity was seen. The extent of increase in activity was dependent on the dilution: the higher the dilution, the greater the increase in active enzyme. By using the more concentrated crude extracts containing 15–25 mg of protein/ml, it was possible to investigate the activation, by using a range of dilutions. Figure 1 shows the relationship between dilution and amine oxidase specific activity after incubation of the samples at 30 °C for 2.5 h. The activation was maximal at 100-fold dilution, and this dilution was used in further experiments with extracts of similar protein concentration. Typically, the specific activity increased 30–50-fold during such an activation, to reach values of $0.05\text{--}0.10 \mu\text{mol/min per mg}$ of protein. When the 10-fold lower amount of amine oxidase protein made by JM109 [pAH15] compared with JM109 [pKC13] is taken into account, the final specific activity ($0.05\text{--}0.10 \mu\text{mol/min per mg}$ of protein) is comparable with the value of $1.5 \mu\text{mol/min per mg}$ of protein found for succinate/ampicillin/IPTG-grown JM109 [pKC13]. This

Figure 2 Protein staining and quinone staining of activated and non-activated amine oxidase

Samples of a crude extract prepared from JM109 [pAH15] (protein = 17.5 mg/ml) diluted 100-fold into 0.1 M sodium phosphate buffer, pH 6.0, and incubated at 30 °C for 180 min, in the absence of EDTA (activated, lane 1) or in the presence of 0.1 mM EDTA (non-activated, lane 2), or incubated at 0 °C instead of 30 °C in the absence of EDTA (non-activated, lane 3), were concentrated before SDS/PAGE as described in the Materials and methods section. For panel (a) each well contained 20 μg of protein, and after electrophoresis the gel was stained with Coomassie Brilliant Blue R. Molecular-mass standards (kDa) are shown on the left. For panel (b) each well contained 680 μg of protein, and after electrophoresis the gel was electroblotted on to nitrocellulose and stained for quinone by the Nitro Blue Tetrazolium/glycinate redox cycling method.

Figure 3 Semicarbazide-sensitivity changes during activation of amine oxidase

A sample of crude extract prepared from JM109 [pAH15] (protein = 16.0 mg/ml) was diluted 100-fold into 0.1 M sodium phosphate buffer, pH 6.0, incubated at 30 °C, and samples were withdrawn at various times up to 250 min for assay of amine oxidase and its semicarbazide-sensitivity. □, Inhibition by 2 mM semicarbazide; ◆, amine oxidase specific activity.

suggests that the amine oxidase present has been converted into essentially the fully active form during the incubation. If 0.1 M EDTA was included in the dilution buffer, the increase in activity was not seen. Similarly, if a 100-fold diluted sample was kept at 0 °C rather than at 30 °C for the duration of the activation experiment, no increase in activity was observed. It seemed that it was the process of activation that was inhibited in the undiluted extract and that this process may involve a metal ion.

To see if this inhibition was due to a dissociable low-molecular-mass compound, 0.5 ml of concentrated crude extract from JM109 [pAH15] was dialysed overnight against 400 vol. of 0.1 M sodium phosphate buffer, pH 6.0. The dialysed extract still did not activate when incubated at 30 °C, but did so when diluted 100-fold before the incubation. Since the inhibitor of activation was not lost on dialysis, it may thus be a high-molecular-mass compound, or a low-molecular-mass compound that is tightly bound to the amine oxidase. The atypical location in the cytoplasm of the amine oxidase produced by JM109 [pAH15] may have resulted in its incorrect folding, and the increase in activity could be due to the refolding of a protein that already had its quinone cofactor. Alternatively, the increase in activity may be due to the formation *de novo* of the quinone cofactor. To investigate these possibilities, samples of the activated protein and the controls were subjected to SDS/PAGE and then electroblotted on to a nitrocellulose membrane for assay of quinone by the redox cycling method [13]. The activated sample stained positively for quinone (Figure 2b, lane 1), whereas samples which had been diluted into buffer containing 0.1 mM EDTA (Figure 2b, lane 2) or diluted and then stored on ice (Figure 2b, lane 3) were both negative. A similar gel stained for protein (Figure 2a) showed that all three JM109 [pAH15] samples contained equivalent amounts of the 80 kDa amine oxidase protein. It seemed that the increase in amine oxidase activity was a consequence of quinone formation.

The activity of the unactivated enzyme was unaffected by 2 mM semicarbazide, an inhibitor of quinoenzymes, but sensitivity to semicarbazide inhibition developed during activation of the enzyme, as shown in Figure 3. The activated enzyme was inhibited by 98 % by 2 mM semicarbazide. This change in sensitivity to semicarbazide is further evidence that a change in the nature of the amine oxidase occurred during the activation process, and is consistent with the view that the increase in activity was due to the formation of a quinone cofactor. Curiously, it seems that the enzyme is able to catalyse amine oxidation, albeit at a very low rate, in the absence of TPQ. The significance of this observation for the reaction mechanism remains to be resolved.

The work reported here describes the conversion of an inactive form of an amine oxidase into its active quinoprotein form. At present it is not possible to exclude the involvement of proteins from the host organism in this process, but the fact that quinone formation occurs in unsupplemented, dilute, dialysed crude extracts suggests that no cofactors are required for the reaction. Clearly, it could be an autocatalytic process, and these possibilities are under investigation. The mechanism and physiological significance of the inhibition that prevents quinone formation in the cytoplasm *in vivo* are also of interest. The inhibitor may serve to prevent the autocatalytic formation of the quinone until the amine oxidase is transported through the cytoplasmic membrane into its normal location in the periplasm, and these aspects also are being studied.

We thank Mrs. D. Langton for synthesis of oligonucleotides and Mrs. U. Gervind-Richards for producing the manuscript. The research was supported by a Wellcome Trust grant, number 035179/1.5.

REFERENCES

- Salisbury, S. A., Forrest, H. S., Cruse, W. B. T. and Kennard, O. (1979) *Nature* (London) **280**, 843–844
- Duine, J. A., Frank, J. and Verweil, P. E. J. (1980) *Eur. J. Biochem.* **108**, 192–197
- Duine, J. A. (1991) *Eur. J. Biochem.* **200**, 271–284
- Janes, S. M., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L. and Klinman, J. P. (1990) *Science* **248**, 981–987
- McIntire, W. S., Wemmer, D. E., Chistoserdov, A. and Lidstrom, M. E. (1991) *Science* **252**, 817–824
- Davidson, V. L. (ed.) (1993) *Principles and Applications of Quinoproteins*, Dekker, New York
- Klinman, J. P. and Mu, D. (1994) *Annu. Rev. Biochem.* **63**, 300–344
- Mu, D., Janes, S. M., Smith, A. J., Brown, D. E., Dooley, D. M. and Klinman, J. P. (1992) *J. Biol. Chem.* **267**, 7979–7982
- Cooper, R. A., Knowles, P. F., Brown, D. E., McGuire, M. A. and Dooley, D. M. (1992) *Biochem. J.* **288**, 337–340
- Parrott, S., Jones, S. and Cooper, R. A. (1987) *J. Gen. Microbiol.* **133**, 347–351
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M. and Gallop, P. M. (1991) *J. Biol. Chem.* **266**, 689–692
- Tabor, S. and Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767–4771
- Kraft, R., Tardiff, J., Kranter, K. S. and Leinwand, L. A. (1988) *Biotechniques* **6**, 544–547
- Cooper, R. A., Jones, D. C. N. and Parrott, S. (1985) *J. Gen. Microbiol.* **131**, 2753–2757
- Suginio, H., Sasaki, M., Azakami, H., Yamashita, M. and Murooka, Y. (1992) *J. Bacteriol.* **174**, 2485–2492
- Konrad, M. (1990) *Nucleic Acids Res.* **18**, 5320
- Azakami, H., Yamashita, M., Roh, J.-H., Suzuki, H., Kumagai, H. and Murooka, Y. (1994) *J. Ferment. Bioeng.* **77**, 315–319