Binding of FAD to 6-hydroxy-D-nicotine oxidase apoenzyme prevents degradation of the holoenzyme

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Expression of the 6-hydroxy-D-nicotine oxidase (6-HDNO) gene from *Arthrobacter oxidans* cloned into *Escherichia coli* showed a marked temperature-dependence. Transformed *E. coli* cells grown at 30 °C exhibited a several-fold higher 6-HDNO activity than did cells grown at 37 °C. This effect did not depend on the promoter used for expression of the cloned gene in *E. coli*, nor was it an effect of 6-HDNO mRNA instability at 37 °C. Studies performed *in vivo* and *in vitro* revealed that an increased susceptibility of apo-6-HDNO to proteolytic attack at 37 °C was responsible for the observed phenomenon. Extracts from cells grown at 37 °C showed on Western blots a decrease in immunologically detectable 6-HDNO polypeptide when compared with extracts from cells grown at 30 °C. The 6-HDNO polypeptide is covalently modified by attachment of the cofactor FAD to a histidine residue. It could be shown that covalent flavinylation of the apoenzyme *in vitro*, i.e. formation of holoenzyme, by incubation of cell extracts with FAD and phosphoenolpyruvate protected the 6-HDNO polypeptide from degradation at 37 °C. Of a variety of proteinase inhibitors tested only the cysteine-proteinase inhibitor L-3-*trans*-carboxyoxiran-2-carbonyl-L-leucylagmatine (E64) prevented degradation, by up to 70 %, of the apoenzyme.

INTRODUCTION

Proteolytic breakdown in *Escherichia coli* of abnormal polypeptides is a well-documented regulatory cellular response (Olden & Goldberg, 1978), and a series of proteinases from *E. coli* have been described (Goldberg *et al.*, 1982). An important role in this is played by ATP-dependent proteinases such as proteinase La (Goff *et al.*, 1984) and proteinase Ti (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987). The natural intracellular substrates for *E. coli* proteinases are, however, known in only a few cases. Thus the λ repressor is specifically cleaved by the RecA proteinase (Roberts & Craig, 1978), and the maturation of membrane and secreted protein precursors involves specific proteinases (Davis & Tai, 1980). The conversion of the A polypeptide of β -galactosidase into the β polypeptide was attributed to an endopeptidase different from proteinase La (McKnight & Fried, 1983), and the purification of a proteinase from *E. coli* that specifically degrades oxidized but not native glutamine synthetase has been described (Roseman & Levine, 1987).

One signal postulated to regulate enzyme turnover by proteolysis is the dissociation of the cofactor from the holoenzyme (Katunuma, 1977). This event was considered to mark the apoenzymes of pyridoxal phosphate-dependent enzymes from rat and yeast (Katunuma *et al.*, 1971a,b; Katunuma *et al.*, 1972) for proteolytic attack by specific proteinases.

We expressed by genetic engineering techniques in *E. coli* the covalently flavinylated enzyme 6-hydroxy-D-nicotine oxidase (6-HDNO) from *Arthrobacter oxidans* (Brandsch *et al.*, 1986). In *E. coli* covalent attachment of the cofactor to the polypeptide does take place and an enzymically active protein indistinguishable from the *A. oxidans* enzyme is formed. The protein expressed in

E. coli has the same specific activity, M_r , N-terminal and C-terminal amino acid sequences and tryptic/chymotryptic peptide pattern as the protein isolated from *A. oxidans* (Brandsch *et al.*, 1987b). Also, the mechanism of formation of holoenzyme by covalent flavinylation of 6-HDNO apoenzyme, a phosphoenolpyruvate-dependent process (Brandsch & Bichler, 1987) is apparently identical in the two organisms.

In the present paper we show *in vitro* and *in vivo* that covalent flavinylation protects the apo-6-HDNO polypeptide from degradation. A temperature-dependent equilibrium between flavinylation and proteolytic degradation of the 6-HDNO apoenzyme seems to determine the amount of holoenzyme present in the cells. At 37 °C less holoenzyme is formed because proteolysis of apoenzyme is faster than flavinylation. At 37 °C apoenzyme can accumulate and flavinylation proceeds because proteolysis is slower at this temperature. This result of these competing processes is a higher enzyme activity in cells grown at 30 °C than in cells grown at 37 °C.

MATERIALS AND METHODS

Chemicals

Diphenyleneiodonium was kindly given by Dr. A. R. Cross (Bristol, U.K.). [14 C]Riboflavin was purchased from Amersham Buchler (Braunschweig, Germany). [14 C]FAD was synthesized from [14 C]riboflavin with the aid of an FAD synthetase preparation kindly given by Dr. A. Bacher, Munich, Germany), and the [14 C]FAD formed was purified by h.p.l.c. after acetone precipitation of the protein. ATP, phosphoenolpyruvate (PEP), phosphoenolpyruvate kinase and FAD were purchased from Boehringer Mannheim (Mannheim, Germany). Isopropyl β -thiogalactoside was purchased from Sigma

Abbreviations used: 6-HDNO, 6-hydroxy-D-nicotine oxidase; PEP, phosphoenolpyruvate.

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(Munich, Germany). Proteinase inhibitors were kindly given by Dr. D. H. Wolf (Freiburg, Germany).

Growth of bacterial cells

E. coli JM101 carrying the 6-HDNO gene on the recombinant plasmid pDB222 (Brandsch *et al.*, 1986) was grown in Yt medium (Maniatis *et al.*, 1982) at 30 °C or 37 °C. In pDB222 the 6-HDNO gene is under the control of the *tac* promoter. Cells harbouring this plasmid were induced during the exponential phase of growth with isopropyl β -thiogalactoside in the presence of 5 μ M-diphenyleneiodonium. Cells were harvested 90 min after induction, pelleted by centrifugation, resuspended in 0.1 M-sodium phosphate buffer, pH 7.0, and disrupted by sonication. After removal at 12000 *g* of cell debris, the S-12 supernatant was dialysed against two changes of 5 litres of phosphate buffer, pH 7.0, at 4 °C, overnight in order to remove low- M_r material. A high-speed supernatant was obtained from the dialysed cell extract by centrifugation at 150000 *g* for 2.5 h at 4 °C. This S-150 supernatant was used in the experiments performed *in vitro*.

Dot-blot hybridization

Total RNA was extracted from *E. coli* JM101/pDB222 cells grown at 30 °C or 37 °C after induction with isopropyl β -thiogalactoside for various times (Aiba *et al.*, 1981). Dot-blot hybridization of labelled random-primer-transcribed 6-HDNO gene DNA to RNA samples was performed according to established methods (Davis *et al.*, 1986).

Assay of 6-HDNO activation

6-HDNO activation assay mixtures consisted of 200 μ l of S-150 supernatant supplemented where appropriate with 5 μ M-FAD and/or 8 mM-PEP. For the specific labelling of the 6-HDNO polypeptide 5 μ M-[14 C]FAD (specific radioactivity 50–60 mCi/mmol) was added. Samples were incubated for various times at 30 °C or 37 °C, and 6-HDNO activity was determined photometrically (Möhler *et al.*, 1972). Western blots and immunological staining of the 6-HDNO polypeptide was performed as described previously (Brandsch *et al.*, 1987a).

RESULTS

Temperature-dependent expression of 6-HDNO in *E. coli in vivo*

When 6-HDNO activity was measured in *E. coli* cells harbouring the 6-HDNO gene on a recombinant plasmid, higher specific activities were found in cells grown at 30 °C as compared with cells grown at 37 °C (Fig. 1a). This effect was independent of the plasmid carrying the 6-HDNO gene or the promoter used for transcription of the gene. The same results were obtained when instead of pDB222 the plasmid constructs pDB121 carrying the 6-HDNO gene under control of its natural *A. oxidans* promoter (Brandsch *et al.*, 1986) or pDB522 carrying the 6-HDNO gene under control of the λ PL promoter (Brandsch *et al.*, 1987a) were used (results not shown). Also, no difference in the amount of 6-HDNO mRNA synthesized at 37 °C as compared with 30 °C was found by dot-blot hybridization (results not shown). Thus the observed difference in expression of the 6-HDNO gene

Fig. 1. 6-HDNO activity and content in *E. coli* cells carrying pDB222 grown at 30 °C and at 37 °C

(a) Freshly inoculated *E. coli* cells carrying pDB222 were grown at 30 °C for 2 h. Then the *tac* promoter inducer isopropyl β -thiogalactoside was added and growth of one culture was continued at 30 °C (Δ) but that of a parallel culture was shifted to 37 °C ($\blacktriangle$). At the time points indicated samples were withdrawn and 6-HDNO activity was determined as described in the Materials and methods section. (b) Cells from samples identical with those in (a) were pelleted by centrifugation and lysed by sonication, and 40 μ g of protein was taken for each time point and analysed on Western blots by reaction with 6-HDNO-specific antibody.

did not reside in the DNA derived from the soil bacterium *A. oxidans* with a growth optimum at 28 °C and cloned into *E. coli* with a growth optimum at 37 °C.

Cell extracts from cells carrying pDB222 were prepared at various times after induction of 6-HDNO expression with isopropyl β -thiogalactoside from cultures grown at 30 °C and 37 °C and were analysed on Western blots by immunostaining. Samples taken from cultures grown at 30 °C showed an increase in the amount of 6-HDNO polypeptide paralleling the increase in 6-HDNO activity. Samples taken from cells grown at 37 °C, however, showed that the amount of 6-HDNO polypeptide reached a certain level after 20 min but did not increase further (Fig. 1b).

Flavinylolation and degradation of 6-HDNO apoenzyme *in vitro*

Isopropyl β -thiogalactoside-induced *E. coli* cells harbouring pDB222 in the presence of diphenyleneiodonium expressed 6-HDNO to over 90 % in its apoform. The apoenzyme could be transformed into 6-HDNO holoenzyme in the presence of FAD and PEP (Brandsch & Bichler, 1987). The formation of the holoenzyme was monitored by the corresponding increase in 6-HDNO activity in the activated S-150 supernatant and by incorporation of [14 C]FAD into the apo-6-HDNO polypeptide. We used this system to analyse *in vitro* the

Fig. 2. Temperature-dependence of 6-HDNO holoenzyme formation

Activation assays with S-150 supernatant were performed at different temperatures as described in the Materials and methods section. At the time points indicated samples were withdrawn for measurement of enzyme activity.

stability of apoenzyme and holoenzyme as a function of flavinylation and temperature.

S-150 samples incubated with FAD and PEP at different temperatures provided the results shown in Fig. 2. With increasing temperature (between 0 °C and 30 °C) an increase in the rate of holoenzyme formation and thus 6-HDNO activity was obtained. The temperature coefficient, Q_{10} , was about 2. At 37 °C, however, after an initial increase, which seemed not to be faster than that at 30 °C, the enzyme activity remained at a constant low level during further incubation.

In order to analyse the fate of the 6-HDNO polypeptide in these samples, we performed the following experiment. S-150 extracts were incubated at 30 °C and 37 °C. After 2, 5, 10 and 15 min, portions of the samples were taken and the 6-HDNO polypeptide was analysed on Western blots by immunological staining with 6-HDNO-specific antibody. A rapid decrease in the amount of 6-HDNO polypeptide in samples incubated at 37 °C as compared with samples incubated at 30 °C was apparent (Fig. 3a). To the remaining S-150 samples [14 C]FAD and PEP were added immediately after the elapsed incubation period of 2, 5, 10 and 15 min. All samples, from 30 °C as well as from 37 °C, were then incubated for 30 min at 30 °C in order to transform the 6-HDNO apoenzyme into holoenzyme. The proportion of apoenzyme present in the samples capable of forming holoenzyme will be reflected in the amount of [14 C]FAD incorporated into the 6-HDNO polypeptide. This can be revealed as radioactive bands of different intensity after SDS/polyacrylamide-gel electrophoresis and fluorography. The results presented in Fig. 3(b) show that incubation of S-150 supernatant containing apo-6-HDNO at 37 °C leads to a decrease in the amount of [14 C]FAD-labelled polypeptide. Thus the analysis of the 6-HDNO polypeptide on Western blots correlates with the amount of apo-6-HDNO transformable into holoenzyme. It indicates that at 37 °C the apo-6-HDNO polypeptide is degraded more

Fig. 3. Temperature-dependent degradation of the 6-HDNO polypeptide in S-150 supernatant

(a) S-150 extracts were incubated at 30 °C and at 37 °C. At the time points indicated samples were withdrawn and analysed on Western blots by 6-HDNO-antibody-mediated immunostaining. (b) Samples taken at the same time points as in (a) were in addition incubated with [14 C]FAD (7 μ M) and PEP (8 mM) at 30 °C and at 37 °C for 30 min and analysed by SDS/polyacrylamide-gel electrophoresis for covalent incorporation of [14 C]FAD.

rapidly than at 30 °C. Since more than 90% of the 6-HDNO polypeptide in the S-150 supernatant analysed is in its apo form, the effect observed is mainly due to degradation of the apoenzyme.

These observations suggested that at 37 °C the proteolytic degradation of the apoenzyme is faster than its flavinylation, and that flavinylation protects the 6-HDNO polypeptide from proteolytic attack. If this assumption is correct, preincubation of the S-150 supernatant at 37 °C before activation should diminish the increase in 6-HDNO activity found after addition of FAD and PEP, because the apoenzyme present in the cell extract should be largely degraded. Activation with FAD and PEP at 30 °C, i.e. formation of the holoenzyme, followed by incubation at 37 °C should not alter the level of enzyme activity in the sample, because the holoenzyme formed is no longer susceptible to proteolytic attack. The results of such experiments are presented in Fig. 4. Incubation of S-150 supernatant with FAD and PEP at 30 °C for 30 min resulted in the enzyme activity shown by Δ symbols in Fig. 4, which remained constant during further incubation at 30 °C. Activation at 30 °C for 30 min followed by incubation at 37 °C did not change the level of enzyme activity ($\blacktriangle$ symbols in Fig. 4), indicating that the holoenzyme formed at 30 °C was not degraded at 37 °C during the incubation period. Activation of S-150 supernatant with FAD and PEP at 37 °C led to a lower level of enzyme activity. This enzyme activity was maintained on further incubation at 30 °C ($\bullet$ symbols in Fig. 4). Preincubation for 30 min at 30 °C before activation, followed by addition of FAD and PEP and incubation at 37 °C ($\blacklozenge$ symbols in Fig. 4) resulted in a lower level of 6-HDNO activity than in the case of direct activation at 37 °C ($\bullet$ symbols in Fig. 4). This finding suggested that at 30 °C part of the 6-HDNO apoenzyme was degraded, although more slowly than at 37 °C. Preincubation at 37 °C followed by activation at 30 °C ($\diamond$ symbols in Fig. 4) showed that almost no

Fig. 4. Effect of incubation at different temperatures of cell extracts containing apo-6-HDNO on the formation of 6-HDNO holoenzyme

Samples of S-150 supernatant were either first activated for 30 min at 30 °C or at 37 °C with FAD and PEP and then shifted to 37 °C or 30 °C, or were preincubated for 30 min without FAD and PEP at 30 °C and at 37 °C and then activated with FAD and PEP at 37 °C or 30 °C. At the time points indicated 6-HDNO activity was determined. Δ , Activation at 30 °C with FAD plus PEP, without temperature shift (control); $\blacktriangle$, activation at 30 °C, shift to 37 °C; $\bullet$, activation at 37 °C, followed by incubation at 30 °C; $\blacklozenge$, preincubation at 30 °C, then activation at 37 °C; $\diamond$, preincubation at 37 °C, then activation at 30 °C.

apoenzyme remained to be transformed into holoenzyme in the presence of FAD and PEP. Addition of PEP or FAD alone to the preincubation sample did not show the protective effect observed when both FAD and PEP were added.

Analysis of the 6-HDNO polypeptide on Western blots by immunological staining parallels the observations made by measuring 6-HDNO activity. Incubation at 30 °C without activation, i.e. incubation of the apo-6-HDNO for 60 min, had only a slight effect on the amount of detectable 6-HDNO as compared with a sample taken from time zero (Fig. 5, lanes 1 and 2, respectively). In samples preincubated at 37 °C before activation, however, almost no 6-HDNO-antibody-reactive material was detectable any more after 60 min of incubation (Fig. 5, lane 3). Samples in which holoenzyme was first formed by incubation with FAD and PEP, and subsequently incubated at 37 °C for 60 min, showed no detectable changes in the amount of 6-HDNO polypeptide present (Fig. 5, lane 4), indicating that the holoenzyme once formed was protected against proteolytic degradation.

Temperature effect on activation of antibody-Sepharose-bound apo-6-HDNO

From the experiments presented it is not possible to differentiate between increased proteolytic activity and

Fig. 5. Western-blot analysis of the 6-HDNO polypeptide from activation assays performed at different temperatures

Samples (40 μ g of protein) from S-150 activation assays were loaded on a SDS/10%-polyacrylamide gel, separated by electrophoresis, blotted on to nitrocellulose and immunologically stained with 6-HDNO-specific antibody. Lane 1, protein from S-150 supernatant incubated at 30 °C for 60 min; lane 2, protein from S-150 supernatant before incubation (time 0); lane 3, protein from S-150 supernatant incubated at 37 °C for 60 min; lane 4, protein from S-150 supernatant first activated with FAD and PEP and then incubated at 37 °C for 60 min.

an increased conformational instability of the apoenzyme at 37 °C. A partial unfolding of the apoenzyme at 37 °C could lead to a higher proteinase-susceptibility. Binding of FAD would stabilize the conformation of the polypeptide against denaturation and thus protect it from proteolytic attack. We prepared antibody-Sepharose-bound apo-6-HDNO and found that under these conditions, where the proteolytic activity was no longer present in the assay mixtures, flavinylation of the apoenzyme was increased at the higher temperature. After 30 min of incubation of antibody-Sepharose-bound apoenzyme with FAD and PEP at 37 °C 205 munits/ml was reached as compared with 133 munits/ml at 30 °C. Thus the velocity of the flavinylation reaction itself is, as would be expected, temperature-dependent. Apo-6-HDNO bound to antibody-Sepharose was re-added to S-150 supernatant, incubated at 37 °C with FAD and PEP, and enzyme activity was measured after 30 min. With these experimental conditions we wanted to test whether bound apo-6-HDNO is still prone to proteolysis. We used an S-150 supernatant preincubated at 37 °C for 60 min, in which endogenous apo-6-HDNO was no longer detectable. Under these conditions, incubation at 37 °C of antibody-Sepharose-bound apoenzyme with FAD and PEP did not prevent formation of holoenzyme. We take this as an indication that apoenzyme bound to antibody is more resistant to proteolysis.

Effect of proteinase inhibitors on 6-HDNO apoenzyme stability

Degradation of apo-6-HDNO by proteinases at 37 °C should be affected by proteinase inhibitors. One well-characterized proteinase in *E. coli* is proteinase La, an ATP-dependent serine proteinase (Goff *et al.*, 1984). Degradation of 6-HDNO apoenzyme was, however, not prevented in the presence of serine-proteinase inhibitors. In addition, S-150 supernatant prepared from a lon⁻ strain, which presumably does not contain proteinase La (kindly provided by Dr. A. Goldberg), transformed with pDB222 showed the same temperature-dependence of 6-HDNO holoenzyme formation. The S-150 supernatant is a dialysed cell extract and thus very low in ATP content.

Addition of ATP, however, did not stimulate breakdown of apo-6-HDNO. Thus proteinase La seems not to be involved in the degradation of apo-6-HDNO. In addition, the following proteinase inhibitors were tested: serine-proteinase inhibitors [di-isopropyl phosphorofluoridate (1 mM) and phenylmethanesulphonyl fluoride (1 mM)], peptide analogue inhibitors [pepstatin (50 µg/ml), chymostatin (50 µg/ml), bestatin (50 µg/ml), leupeptin (100 µg/ml) and antipain (100 µg/ml)], metal-ion chelators [*o*-phenanthroline (1 mM), EGTA (10 mM) and EDTA (10 mM)] and cysteine-proteinase inhibitors [L-3-*trans*-carboxyoxiran-2-carbonyl-L-leucylagmatine (E64) (50 µg/ml), HgCl₂ (10 µM) and *p*-hydroxymercuribenzoate (10 µM)] (Katunuma *et al.*, 1983; Achstetter *et al.*, 1984). To test their effect proteinase inhibitors were added to S-150 samples and incubated for 1 h at room temperature. Control samples incubated without inhibitor showed that during this incubation period the apoenzyme was not degraded, since addition of FAD and PEP resulted in the expected increase in enzyme activity. After this incubation, FAD and PEP were added and parallel cultures were incubated at 30 °C and 37 °C for 30 min. At the end of the incubation period enzyme activity was determined. The activity found at 37 °C was expressed as percentage of the activity measured at 30 °C, which was set at 100 %. If the proteinase inhibitor protected the apoenzyme from degradation at 37 °C, subsequent incubation with FAD and PEP should result in the same enzyme activity as that found in samples incubated at 30 °C. No protection was found with various inhibitors tested with the exception of L-3-*trans*-carboxyoxiran-2-carbonyl-L-leucylagmatine. Incubation of samples at 37 °C in the presence of this inhibitor resulted in 70 % of the enzyme activity found in control samples incubated at 30 °C.

Activation assays performed with extracts of cells grown at different temperatures

Activation assays with S-150 extracts prepared from isopropyl β-galactoside-induced cells grown at 30 °C and 37 °C in the presence of diphenyleiodonium showed the same behaviour as S-150 extracts incubated *in vitro* at these temperatures. Cell extracts from cells grown at 30 °C could be activated with FAD and PEP and thus contained apoenzyme. However, no activation was possible with cell extract obtained from cultures grown at 37 °C. *In vivo* at 37 °C, as shown for cell extracts *in vitro*, the apoenzyme was rapidly degraded and no longer available for 6-HDNO holoenzyme formation (results not shown).

DISCUSSION

In eukaryotes a specific relationship between holoenzyme, apoenzyme and proteolytic degradation was discussed and established several years ago for pyridoxal phosphate-dependent enzymes of rat and yeast. In rats kept on pyridoxal-deficient diet, Katunuma *et al.* (1971a, b) found a rapid and marked decrease in ornithine carboxylase in the small intestine. Pyridoxal phosphate protected pyridoxal enzymes from inactivation. Proteolytic degradation of pyridoxal phosphate-containing enzymes was postulated to require first loss of the prosthetic group, followed by the action of a specific proteinase upon the apoenzyme. The product of this degradation is then further degraded by non-specific

proteinases (Katunuma, 1977). Similar proteolytic enzymes were described in yeast for tryptophan synthase (Schött & Holzer, 1974). These enzymes inactivated the pyridoxal phosphate-dependent apoenzyme and were inhibited by pyridoxal phosphate and serine. The apoprotein of NAD-dependent enzymes was also shown to be inactivated by specific proteinases, both in rat (Katunuma *et al.*, 1971a, b) and in yeast (Hemmings *et al.*, 1980).

In the present paper we show that in a prokaryotic system the covalent binding of flavin cofactor to the apoenzyme stabilizes the protein against proteolytic attack. The stabilization against proteolysis of the 6-HDNO polypeptide by flavinylation could be one function of this, as yet poorly understood, covalent modification of enzymes.

The competition between flavinylation and proteolysis of the apoenzyme may explain the higher specific 6-HDNO activity at 30 °C as compared with that at 37 °C. Proteolytic degradation at 30 °C is apparently slow, and hence a large proportion of the apoenzyme can be flavinylated and transformed into the enzymically active state.

We could show no activation of proteases at 37 °C in the S-150 supernatant. Cell extracts preincubated at 37 °C and then added to S-150 supernatant containing apo-6-HDNO did not induce the degradation of the apo-form in these samples when incubated at 30 °C. Thus either proteolysis seems to be slower at the lower temperature, or the apo-6-HDNO polypeptide is partially denatured at the higher temperature, becoming a better substrate for proteolysis, or both. The 6-HDNO holoenzyme, however, becomes denatured only above 56 °C (Hinkkanen, 1984).

On Western blots no low-*M_r* degradation products of the 6-HDNO polypeptide were observed. This could be due to a very fast breakdown of the apoenzyme once proteolysis is initiated or because the breakdown products are no longer recognized by the 6-HDNO antibody. We also emphasize that on SDS/polyacrylamide-gel electrophoresis with [¹⁴C]FAD-labelled 6-HDNO polypeptide (Fig. 3b) no formation of labelled products of lower *M_r* than that of the 6-HDNO polypeptide was observed. This finding is in agreement with our assumption that flavinylation of the apoenzyme stabilizes the polypeptide against proteolytic attack. We consider that the difference in enzyme activity found between cells grown at 30 °C and 37 °C reflects the situation found *in vitro* with cell extracts and that the lower 6-HDNO activity in cells grown at 37 °C is not due to heat-shock induction of proteinases such as proteinase La.

The extent of flavinylation of the apo-6HDNO depends also on the available PEP. A more rapid utilization at 37 °C of PEP by other pathways would favour proteolytic degradation of the apoenzyme.

Thus the actual concentration of holoenzyme at a certain growth temperature seems to depend on the competition between the processes leading to flavinylation and proteolysis of the apoenzyme.

What proteolytic pathway, specific or not, might be responsible for apo-6-HDNO degradation remains as yet unknown. An ATP-independent cysteine proteinase seems to be involved. It has to be shown in future work with purified apoenzyme whether a specific proteinase can be identified as being responsible for the degradation of the unflavinylated 6-HDNO polypeptide.

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