Riboflavin-dependent expression of flavoenzymes of the nicotine regulon of *Arthrobacter oxidans*

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In cells of an *Arthrobacter oxidans* riboflavin-dependent mutant the specific activity of the DL-nicotine-inducible *nic*-regulon enzymes nicotine dehydrogenase (NDH, EC 1.5.99.4), 6-hydroxy-L-nicotine oxidase (6-HLNO, EC 1.5.3.5) and 6-hydroxy-D-nicotine oxidase (6-HDNO, EC 1.5.3.6) was shown to be dependent on the supply of the vitamin in the growth medium. Experiments designed to identify at which level riboflavin directs the biosynthesis of these flavoenzymes revealed that the steady-state levels of enzyme protein analysed on Western blots correlated directly with riboflavin supply from the minimal concentration of 0.5 μ M-riboflavin required for growth up to 8 μ M-riboflavin. Mutant cells grown at the higher riboflavin concentration showed on dot-blots increased levels of RNA which hybridized to 32 P-labelled probes derived from the *nic*-regulon genes. When cells grown at 2 μ M-riboflavin were shifted to 8 μ M-riboflavin, 6-HDNO expression increased as indicated by elevated enzyme and RNA levels. When the rates of synthesis of the 6-HDNO and 6-HLNO polypeptides after DL-nicotine induction was analysed in cells grown at 0.5 μ M and 8 μ M-riboflavin, only cells grown at the higher riboflavin concentration showed on Western blots an accumulation of the polypeptides. No 6-HDNO or 6-HLNO polypeptide was identified in cell extracts from cells grown on 0.5 μ M-riboflavin. Pulse-chase experiments with [35 S]methionine showed that 6-HDNO- and 6-HLNO synthesis was prevented in cells grown at the low riboflavin concentration. The absence of detectable enzyme levels seemed not to be caused by proteolytic breakdown. Incubation *in vitro* of apo-6HDNO with low- or high-riboflavin-grown-cell extracts showed no increased proteolytic activity in 0.5 μ M-riboflavin-grown cells. From these results it is concluded that riboflavin supply co-regulates the expression of the *nic*-regulon genes at the level of transcription and/or mRNA turnover.

INTRODUCTION

In bacteria, inorganic cofactors co-regulate the expression of their apoenzymes. Thus the synthesis of enzymes containing molybdenum (Iuchi & Lin, 1987), zinc (Doyle & Arp, 1986) or selenium (Boursier *et al.*, 1988) was shown to be dependent on the supply of the metal ions. Only few data are, however, available on the relationship between the supply of organic cofactors and the biosynthesis of the corresponding apoenzymes.

Herein the role played by riboflavin in the biosynthesis of flavoenzymes was investigated. Our experimental model consists of the enzymes of the nicotine regulon (*nic*-regulon) of *Arthrobacter oxidans* (Decker & Bleeg, 1965). The metabolic pathway of DL-nicotine utilization by this soil bacterium starts with nicotine dehydrogenase, a Mo-pterin-Fe-S cluster- and FAD-dependent enzyme composed of three unequal polypeptides (Freudenberger *et al.*, 1988). The next enzymic step in the pathway is catalysed by two enantiozymes: 6-hydroxy-L-nicotine oxidase (6-HLNO, EC 1.5.3.5) is a FAD-dependent enzyme composed of two identical subunits (Hinkkanen *et al.*, 1983) and 6-hydroxy-D-nicotine oxidase (6-HDNO EC 1.5.3.6) is a monomeric enzyme. 6-HDNO is covalently modified by the attachment of FAD to a histidine residue of the polypeptide chain (Brühmüller *et al.*, 1972). The NDH and 6-HLNO genes have been cloned (S. Grether-Beck & R. Brandsch, unpublished work), and the 6-HDNO gene has been cloned and sequenced (Brandsch *et al.*, 1987b).

The effect of changes in cofactor supply on expression of these enzymes was studied with the aid of a riboflavin-dependent *A. oxidans* mutant. This mutant was described by Hamm & Decker (1978), and it was shown that, below a certain riboflavin level but in the presence of the inducer DL-nicotine, no 6-HDNO or 6-HLNO activity could be detected in these cells, although cell

growth was normal. From this observation it was concluded that apoenzyme synthesis was dependent on cofactor supply. However, there remained several questions regarding this interesting mode of enzyme regulation. In principle, enzyme levels may be regulated by transcriptional, translational or post-translational events. With the cloned *nic*-regulon genes we were in a position to test the effect of riboflavin supply on the expression of these genes directly by RNA dot-blot hybridization. An increased proteolytic degradation of apoenzymes in conditions of limited cofactor supply could account for the observed absence of enzyme activity. Therefore we analysed the rate of enzyme synthesis and enzyme degradation on Western blots and in pulse-chase experiments with the aid of specific antisera. The results presented here provide evidence that flavin supply co-regulates expression of the genes of these enzymes at the mRNA level.

MATERIALS AND METHODS

Strains and plasmids

The *A. oxidans* wild-type strain and the riboflavin-requiring mutant, *A. oxidans* rf, were described previously (Eberwein *et al.*, 1961; Hamm & Decker, 1978). *A. oxidans* rf cells were grown in citrate medium (Hamm & Decker, 1978) supplemented with 0.05% yeast extract. A 10% inoculum from a culture grown overnight with 2 μ M-riboflavin was used to start cultures in media of lower riboflavin concentration. A riboflavin concentration of 0.5 μ M was found to be the minimum at which these cultures reached the same cellular protein content (per ml) after 24 h of growth at 28 °C as those grown at higher riboflavin concentrations. The mutant riboflavin-dependent *Escherichia coli* strain was kindly provided by A. Bacher.

Abbreviations used: 6-HDNO, 6-hydroxy-D-nicotine oxidase; 6-HLNO, 6-hydroxy-L-nicotine oxidase; NDH, nicotine dehydrogenase; Gro-3-P, glycerol 3-phosphate; *nic*-regulon, nicotine regulon; IPTG, isopropyl β -thiogalactoside.

The 6-HdNO gene is carried on the expression plasmid pDB222 (Brandsch *et al.*, 1987a). For random primer labelling of internal 6-HdNO gene sequences, plasmid pDM1217 (L. Mauch & R. Brandsch, unpublished work) was used. It carries a 960 bp *PvuII*-*NdeI* DNA fragment derived from the coding sequence of the 6-HdNO gene, which was cloned into the corresponding sites of plasmid pTZ19R (Pharmacia, Freiburg, Germany). The genes of the three NDH subunits are located on an approx. 6.9 kb *EcoRI* restriction fragment derived from pA01 (Brandsch & Decker, 1984). On the same fragment the coding sequence for the *N*-terminal 30 amino acids of the 6-HLNO is located (S. Grether-Beck & R. Brandsch, unpublished work). This fragment was cloned into the *EcoRI*-restriction-endonuclease site of plasmid pKK-223-3 (Pharmacia) and used for the preparation of a random primer ^{32}P -labelled probe specific for NDH/6-HLNO gene transcripts.

Preparation of cell extracts

A. oxidans rf cells were grown at different concentrations of riboflavin as indicated in the legends to the Figures. The *nic*-regulon genes were induced by the addition of 0.05% DL-nicotine in the growth medium. DL-Nicotine was added either from the beginning or to 16 h cultures, and samples were withdrawn at the time intervals indicated in the legends to the Figures. Cells were harvested by centrifugation, and S-150 cell extracts were prepared as described by Brandsch & Bichler (1987). The protein contents of these cell extracts were determined as described by Bradford (1976). pDB222-transformed *E. coli* rf cells were grown overnight at 30 °C in Yt-medium (Maniatis *et al.*, 1982) supplemented with various riboflavin concentrations as indicated in the legends to the Figures. From these cultures fresh Yt-medium containing riboflavin was inoculated 1:10 and growth continued for 3 h. Then the inducer isopropyl β -thiogalactoside (IPTG) was added and, after an additional 5 h, the cells were harvested by centrifugation, lysed by sonication and S-150 cell extracts prepared as described by Brandsch & Bichler (1987).

Determination of enzyme activity

Enzyme activities of 6-HdNO, 6-HLNO and NDH were measured photometrically in S-150 extracts by the method of Brühmüller *et al.* (1972). Conversion of apo-6-HdNO into holoenzyme was accomplished in the presence of 7 μM -FAD and 8 mM-glycerol 3-phosphate (Gro-3-P) (Brandsch & Bichler, 1989).

Immunological methods

Polyclonal antisera against 6-HdNO and 6-HLNO were raised in rabbits and the IgG fraction isolated as described by Nagursky *et al.* (1988). The antiserum preparation against NDH recognizes all three polypeptide subunits of the holoenzyme and was kindly given by Dr. H. Nagursky (Biochemical Institute, Freiburg, Germany). Western blots and immunostaining were performed with a commercial kit according to the instructions of the manufacturer (Sigma, Munich, Germany).

Dot-blot hybridizations

RNA was extracted from DL-nicotine-induced *A. oxidans* wild-type (w) or rf cells grown at different riboflavin concentrations by the method of Aiba *et al.* (1981). As a control, RNA extracted from *A. oxidans* cells grown in the presence of 8 μM -FAD but without the inducer DL-nicotine was used. Application of RNA samples to nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany), prehybridization and hybridization of these membranes with ^{32}P -labelled probes were carried out by standard methods (Maniatis *et al.*, 1982; Feinberg & Vogelstein, 1983;

Davis *et al.*, 1986). Gene-specific hybridization probes were derived from the plasmids described above. After hybridization the washed and dried filters were exposed to Fuji X-Omat film in the presence of an intensifying screen.

Pulse-chase experiments

DL-Nicotine was added to 16 h *A. oxidans* cultures in citrate medium and induction allowed to proceed for 3 h. Then 100 μCi of [^{35}S]methionine (Amersham Buchler, Braunschweig, Germany) was added to 20 ml of *A. oxidans* culture and incubation continued for 15 min. After this time the cells were harvested by centrifugation, washed once with citrate medium and resuspended in citrate medium supplemented with 1 mM-methionine. Incubation was continued for the time intervals indicated in the legends to the Figures, after which the cells were collected by centrifugation, lysed by sonication, and the 6-HdNO and 6-HLNO polypeptides were precipitated with specific antiserum and Protein A-Sepharose (Pharmacia).

RESULTS

Enzyme-protein levels in stationary-phase *A. oxidans* rf cells as a function of riboflavin supply

NDH, 6-HLNO and 6-HdNO, the first three enzymes of the pathway induced by DL-nicotine in *Arthrobacter oxidans*, have been isolated and characterized (Hinkkanen *et al.*, 1983; Freudenberg *et al.*, 1988). They show highest specific activity in stationary-phase cells, although their induction pattern differs. Synthesis of NDH and 6-HLNO starts in exponential-phase cells, but 6-HdNO is expressed only in stationary phase (Decker & Blegg, 1965). Therefore cells grown for 24 h at various riboflavin concentrations were used for the experiments described here. The levels of the *nic*-regulon enzymes and RNA in these cells represent steady-state levels at this particular growth stage.

A positive correlation between riboflavin concentration in the growth medium and enzyme protein in the cell extract was found for all three enzymes (Fig. 1). Western blots developed with NDH antiserum showed, under the experimental conditions employed, only the subunits of M_r 95000 and 31000 (Fig. 1,

Fig. 1. Riboflavin-dependent expression of *nic*-regulon flavoenzymes analysed on Western blots

Equal amounts of lysate (30 μg of protein) prepared from *A. oxidans* rf cells grown for 24 h at various riboflavin concentrations were separated by SDS/PAGE, blotted on to nitrocellulose membranes and immunologically stained as described in the Materials and methods section. NDH, Western blot allowed to react with NDH-specific antiserum; the M_r values of the NDH subunits are indicated as well as those of the two cross-reacting polypeptides ('X'). The migrations of M_r standards are indicated on the right-hand side of the panel. The 17000- M_r subunit was not revealed on the 7.5%-polyacrylamide gels employed in these experiments. 6-HLNO, Western blot allowed to react with 6-HLNO-specific antiserum; the arrow indicates the position of 6-HLNO polypeptide. 6-HdNO, Western blot allowed to react with 6-HdNO-specific antiserum; the arrow indicates the position of 6-HdNO polypeptide.

'NDH'). The NDH-antiserum preparation recognizes besides the NDH subunits two main cross-reacting polypeptides (Fig. 1, 'NDH', 'X'). These polypeptides do not belong to the enzymes of the *nic*-regulon (S. Grether-Beck & R. Brandsch, unpublished work) and, in contrast with the NDH subunits, did not show riboflavin-dependent expression. It is still uncertain which of the NDH subunits bears the FAD moiety, as only one FAD molecule is found per holoenzyme molecule (Freudenberg *et al.*, 1988). However, as shown in Fig. 1 ('NDH'), both NDH subunits, the M_r -95000 as well as the M_r -31000 one, exhibited a similar riboflavin-dependent expression. These genes may be co-ordinately regulated and transcribed into a polycistronic message or the mRNA may exhibit an increased turnover in conditions of riboflavin deficiency. Specific enzyme activities determined in S-150 cell extracts paralleled the increase in enzyme-protein levels as revealed on Western blots (results not shown).

Riboflavin-dependent RNA levels of *nic*-regulon-genes in stationary-phase *A. oxidans* cells

The dependence of RNA levels of the *nic*-regulon genes on riboflavin supply was investigated by dot-blot hybridization with RNA samples isolated from *A. oxidans* cells grown at

Fig. 2. Riboflavin-dependent synthesis of *nic*-regulon-specific RNA

Equal amounts of RNA (10 μg) extracted from *A. oxidans* rf cells grown with 2 μM -riboflavin (lane 2), 8 μM -riboflavin (lane 8) and 8 μM -riboflavin, but in the absence of DL-nicotine (lane 0) were applied per dot. They were hybridized with a random-primer-labelled probe derived from NDH/6-HLNO and 6-HdNO gene sequences. A random-primer-labelled probe derived from a pA01 DNA sequence not belonging to the *nic* regulon was used as control. (b) Quantification of dot sizes obtained on X-ray films exposed to dot-blots. RNA (4, 8 and 16 μg) extracted from cells grown under inducing conditions at 2 μM -riboflavin (Δ , $\blacktriangle$) and 8 μM -riboflavin ($\square$, $\blacksquare$) and from cells grown at 8 μM -riboflavin but without DL-nicotine ($\circ$, $\bullet$) were applied to nitrocellulose membranes and hybridized with random primer-labelled NDH/6-HLNO- and 6-HdNO-gene-specific sequences

various riboflavin concentrations. Random primer-transcribed ^{32}P -labelled DNA sequences derived from *nic*-regulon genes as hybridization probes were employed (see the Materials and methods section).

Fig. 2 presents the results obtained by hybridizing these probes to RNA extracted from *A. oxidans* cells grown under inducing conditions in the presence of 2 μM - and 8 μM -riboflavin respectively. An increase in the intensity of the hybridization signal with RNA from cells grown at the higher riboflavin concentration was found with both probes (Figs. 2a and 2b). Hybridization of the same filters with a random-primer-labelled probe derived from pA01 DNA sequences not related to the *nic*-regulon genes showed no riboflavin-dependent variation in the intensity of the hybridization signal (Fig. 2a, control).

If riboflavin acted as a co-inducer, cells grown at a riboflavin concentration suboptimal for induction should show an increase in the level of enzyme activity and specific RNA when the riboflavin concentration in the medium is raised. To test this assumption, *A. oxidans* rf cells were grown under inducing conditions at the suboptimal concentration of 2 μM -riboflavin for 16 h. Then one part of the original culture was allowed to continue growth at 2 μM -riboflavin, one part was supplemented with riboflavin to 8 μM , and one part was supplemented with riboflavin to 8 μM and with rifamycin (40 $\mu\text{g}/\text{ml}$). All three cultures were grown for an additional 4 h. Subsequently the cells were harvested and 6-HdNO enzyme activity and 6-HdNO RNA levels were determined. Both the specific enzyme activity and 6-HdNO RNA levels increased in cells supplemented with the higher riboflavin concentration (results not shown).

Rate of enzyme and RNA synthesis in cells grown at different riboflavin concentration

To determine the rate of enzyme synthesis, we used the experimental design illustrated in Fig. 3(a). Cells were grown at

Fig. 3. Rate of 6-HdNO- and 6-HLNO-polypeptide and 6-HdNO-specific RNA synthesis as a function of riboflavin supply

A. oxidans wild-type (w) or rf cells grown for 16 h at 0.5 μM - or 8 μM -riboflavin were induced with DL-nicotine and samples withdrawn at time intervals as indicated (a). The proteins of cell lysates prepared from these samples were separated by SDS/PAGE and immunologically stained with 6-HdNO- and 6-HLNO-specific antibody (b). Dot-blots prepared with RNA extracted from samples taken at the indicated time points were hybridized with a 6-HdNO-gene-specific ^{32}P -labelled probe (c).

a constant riboflavin concentration under non-inducing conditions into stationary phase (16 h) and then induced with DL-nicotine. Samples were withdrawn at hourly intervals and analysed for protein and RNA content. Cells grown at $0.5 \mu\text{M}$ -riboflavin showed after 16 h of growth the same absorbance as those grown at $8 \mu\text{M}$ -riboflavin. In both the wild-type (w) and cells grown at $8 \mu\text{M}$ -riboflavin synthesis of the polypeptides can be monitored soon after induction (Fig. 3; 6-HdNO and 6-HLNO). At $0.5 \mu\text{M}$ -riboflavin, no accumulation of 6-HdNO or 6-HLNO polypeptide could be detected within 4 h after DL-nicotine induction (Fig. 3b). RNA samples were hybridized only to the 6-HdNO probe because this probe has a high specificity for the 6-HdNO-gene-transcribed sequences. There is a pronounced difference in the induction of 6-HdNO-gene expression between cells grown at $8 \mu\text{M}$ - and those grown at $0.5 \mu\text{M}$ -riboflavin (Fig. 3, 6-HdNO RNA). The 6-HdNO RNA level in $8 \mu\text{M}$ -riboflavin-grown cells reached its maximum approx. 4 h after induction (Fig. 3c). Cells at $0.5 \mu\text{M}$ -riboflavin showed a slight increase in the RNA level upon induction, which, however, remained very low (Fig. 3c).

Synthesis and turnover of 6-HdNO and 6-HLNO polypeptides analysed by pulse-chase experiments

The riboflavin-dependent expression of the *nic*-regulon enzymes could be wrongly indicated if apoenzymes formed under suboptimal riboflavin supply were quickly proteolytically degraded. This possibility was investigated by pulse-chase experiments. A schematic representation of the strategy of these experiments is shown in Fig. 4(a). Cells grown for 16 h were induced with DL-nicotine for 3 h, followed by a 15 min pulse with [^{35}S]methionine. Subsequently the cells were washed and chased with 1 mM unlabelled methionine for the times indicated. The analysis of the immunoprecipitates is presented in Fig. 4(b). No 6-HdNO or 6-HLNO-specific immunoprecipitate was recovered from cells grown at $0.5 \mu\text{M}$ -riboflavin. A faint band visible in these precipitates represents a cross-reacting polypeptide migrating on PAGE slightly faster than the 6-HdNO-polypeptide. This is revealed by the immunoprecipitates obtained from $8 \mu\text{M}$ -riboflavin-grown cells (Fig. 4b, '8'). At $8 \mu\text{M}$ -riboflavin and in wild-type cells, both polypeptides, 6-HdNO and 6-HLNO, were actively synthesized (Fig. 4, '8' and 'w'). The difference in the amount of incorporation of [^{35}S]methionine between the two proteins reflects the higher level of expression of the 6-HLNO gene. There was no change in the pattern of proteins precipitated with 6-HdNO and 6-HLNO antibody when cells grown and pulse-labelled in medium containing $8 \mu\text{M}$ -riboflavin were switched during the chase to a medium with $0.5 \mu\text{M}$ -riboflavin (Fig. 4, '8' and '0.5').

Apparently, at $0.5 \mu\text{M}$ -riboflavin the synthesis of the 6-HdNO- and the 6-HLNO-polypeptide was prevented. The proteins synthesized at $8 \mu\text{M}$ -riboflavin and in wild-type cells were not detectably degraded during the chase period.

Rate of apo-6-HdNO degradation *in vitro*

The 6-HdNO gene can be overexpressed in *E. coli* cells transformed with the expression plasmid pDB222. Such cells grown in the presence of diphenyliodonium accumulate 6-HdNO polypeptide to over 90 % in its apo form (Brandsch & Bichler, 1987). This apoenzyme can be converted in cell extracts into holoenzyme in the presence of [^{14}C]FAD and Gro-3-P (Brandsch & Bichler, 1989). The radioactive label incorporated covalently into the 6-HdNO-polypeptide can be revealed by fluorography and is a measure of the amount of apoenzyme present (Brandsch & Bichler, 1989). An *E. coli* cell extract containing apoenzyme was mixed 1:1 with an *A. oxidans* extract from nicotine-induced cells grown at either $0.5 \mu\text{M}$ - or $8 \mu\text{M}$ -

Fig. 4. Analysis of riboflavin-dependent synthesis and proteolytic stability of the 6-HdNO- and 6-HLNO polypeptides by pulse-chase experiments

A. oxidans wild-type (w) or rf cells grown for 16 h at $0.5 \mu\text{M}$ - or $8 \mu\text{M}$ -riboflavin were induced for 3 h with DL-nicotine (DL-Nic). Then a 15 min pulse with [^{35}S]methionine ([^{35}S]Met) was applied followed by a chase with unlabelled methionine (Met) and samples were withdrawn at the time points indicated (a). The 6-HdNO and 6-HLNO polypeptides were precipitated from cell extracts prepared from these samples with specific antibody and Sepharose A and analysed by SDS/PAGE, followed by fluorography, as described in the Materials and methods section (b). Samples shifted after the [^{35}S]methionine pulse from $8 \mu\text{M}$ - to $0.5 \mu\text{M}$ -riboflavin in the growth medium are indicated by '8/0.5'.

riboflavin. The activation assays were performed at 30°C for 30 min with $10 \mu\text{M}$ -[^{14}C]FAD and 8 mM -Gro-3-P. The radioactivity incorporated during this time and the 6-HdNO-activity measured in parallel samples was taken as 'point zero'. The rate of apoenzyme degradation was determined in samples pre-incubated at 30°C for 30 and 60 min before incorporation of [^{14}C]FAD and formation of enzymically active holoenzyme respectively. Thus the total time of incubation of these samples at 30°C amounted to 60 and 90 min respectively. A control sample was included where 0.1 M -phosphate buffer, pH 7.2, instead of *A. oxidans* extract was added to *E. coli* cell extracts containing apoenzyme. The radioactivity (Fig. 5b) incorporated during the 30 min of the activation assay and the enzyme activity measured in these samples was taken as a reference.

From the intensity of the [^{14}C]FAD-labelled 6-HdNO polypeptide bands on the fluorogram (Fig. 5b) it may be concluded that a slow degradation of apoenzyme during the period of incubation at 30°C took place. However, there is no difference between samples incubated with extracts from *A. oxidans* cells grown at $0.5 \mu\text{M}$ - or $8 \mu\text{M}$ -riboflavin. Samples incubated with the $8 \mu\text{M}$ -riboflavin-grown-cell extract showed higher enzyme activity (Fig. 5a), since these cell extracts themselves contain a certain amount of 6-HdNO activity. The slow degradation of the apoenzyme *in vitro* revealed by these experiments is insufficient to explain the lack of 6-HdNO-polypeptide in $0.5 \mu\text{M}$ -riboflavin-grown cells. The 10 % holoenzyme activity present in the samples did not change during the incubation period (results not shown). This was in agreement with previous results obtained in *E. coli*, which showed that the 6-HdNO holoenzyme is more stable than the apoenzyme in cells transformed with the 6-HdNO gene (Brandsch *et al.*, 1989).

Fig. 5. Determination of 6-HdNO apoenzyme stability in cell extracts prepared from *A. oxidans* rf cells grown at limiting and optimal riboflavin supply

E. coli cell extracts containing high amounts of 6-HdNO apoenzyme were prepared as described in the Materials and methods section and mixed 1:1 with extracts prepared from *A. oxidans* rf cells grown at 0.5 μM- or 8 μM-riboflavin respectively. Samples (50 μl) were incubated at 30 °C for the time intervals indicated, and holoenzyme was formed in the presence of [¹⁴C]FAD and Gro-3-P (see the Materials and methods section). Samples were analysed for specific 6-HdNO activity (a) or, in parallel samples, the proteins were separated by SDS/PAGE and the [¹⁴C]FAD-labelled 6-HdNO polypeptide identified by fluorography (b). The amount of 6-HdNO activity and the intensity of the [¹⁴C]FAD-labelled 6-HdNO band on the fluorogram is a measure of the amount of apoenzyme present in the cell extracts. K represents the level of 6-HdNO enzyme activity and [¹⁴C]FAD incorporation obtained in the presence of Gro-3-P in an *E. coli* extract mixed 1:1 with 0.1 M-phosphate buffer, pH 7.2.

Fig. 6. Riboflavin-dependent 6-HdNO expression in a riboflavin-requiring *E. coli* strain from the recombinant plasmid pDB222

Determination of 6-HdNO specific activity in cell extracts from pDB222 transformed *E. coli* rf cells and the identification of the 6-HdNO polypeptide on Western blots with 6-HdNO-specific antibody was performed as described in the Materials and methods section. (a) 6-HdNO activity; (b) levels of 6-HdNO polypeptide as revealed on Western blots.

Riboflavin-dependence of 6-HdNO-expression in an *E. coli* riboflavin-requiring strain

The riboflavin-requiring mutant *E. coli* strain harbouring pDB222 was grown in the presence of IPTG at various riboflavin concentrations, and cell extracts prepared as described in Materials and methods section were analysed for specific 6-HdNO activity and on Western blots for the amount of 6-HdNO polypeptide present. The results of this analysis are presented in Fig. 6. In contrast with the situation found in *A. oxidans*, there is only a weak correlation between enzyme activity, the level of 6-HdNO polypeptide detected on Western blots and riboflavin supply. At low riboflavin concentrations, more of the 6-HdNO polypeptide appeared to be degraded than at higher riboflavin concentrations, since at these lower concentrations several protein bands reacting with 6-HdNO antibody were revealed (Fig. 6b). This could also account for the lower specific enzyme activity measured at these riboflavin concentrations. The synthesis of the 6-HdNO polypeptide in *E. coli* cells grown at low concentrations of riboflavin results in a high amount of apoenzyme which can be transformed *in vitro* in cell extracts into holoenzyme by incubation with FAD and Gro-3-P. Fig. 6(a) presents the total amount of enzyme activity measured in cell extracts after the transformation of apoenzyme into holoenzyme. From the expression of apoenzyme in *E. coli* cells, it also follows that the translation of the 6-HdNO-polypeptide is not prevented by the lack of cofactor. This does not exclude the possibility that the cofactor is co-translationally inserted into that part of the 6-HdNO polypeptide present as holoenzyme (Brandsch *et al.*, 1987a).

DISCUSSION

The experimental results presented here suggest that the effect of diminished riboflavin supply on the biosynthesis of the *nic*-regulon enzymes in *A. oxidans* rf cells is an effect at the mRNA level. The mRNA levels of the *nic*-regulon genes in such cells are reduced when compared with cells grown at saturating riboflavin concentrations, and when cells grown under inducing conditions at low riboflavin concentration are supplemented with the vitamin, an increase in 6-HdNO-specific RNA can be observed. These findings argue for a control by the vitamin at the

transcriptional level or for an increase in mRNA turnover in cells supplied with a suboptimal riboflavin concentration.

A shortage of cofactor could lead to the accumulation of apoenzyme, which in turn is rapidly degraded by proteolysis. Our pulse-chase experiments and the direct measurement of 6-HdNO apoenzyme degradation argue against this explanation. Degradation of apoenzyme takes place, but not at a rate sufficient to explain the complete absence of 6-HdNO and 6-HLNO polypeptides in cells grown and induced with DL-nicotine at the limiting riboflavin concentration. Although it was mainly the biosynthesis of 6-HdNO and 6-HLNO which was investigated, taking into consideration the results on the steady-state levels of the *nic*-regulon enzymes in 24 h cells, it is considered that the correlation between riboflavin supply and enzyme synthesis applies also to the NDH polypeptides.

A correlation between riboflavin supply and 6-HdNO synthesis was not found in *E. coli* rf cells transformed with the 6-HdNO gene. This could be due to the fact that the gene is expressed from a multicopy plasmid and an artificial promoter. Under these conditions, factors involved in the mediation of the riboflavin effect may be titrated out. How specific this regulation is for *A. oxidans* cells remains to be established.

In other bacterial systems an effect of riboflavin on enzyme expression was demonstrated with a riboflavin-requiring *Bacillus subtilis* mutant by Bacher *et al.* (1973). They showed that the synthesis of riboflavin synthase was regulated by feedback repression exerted by riboflavin. Regulation of expression by the free FAD pool was recently demonstrated for riboflavin kinase and FMN adenylyltransferase in yeast (Brooke *et al.*, 1986).

The correlation between flavin concentration and gene expression within the *nic*-regulon differs from the situation described above in that these genes are not involved in the biosynthesis of the cofactor. In our experimental model the biosynthesis of flavoenzyme apoproteins appeared to be controlled by the level of available cofactor. The sensor in this regulation could be the intracellular free FAD pool. It was shown previously that the intracellular free FAD pool increases in this mutant with the external riboflavin concentration and that 6-HdNO activity paralleled this increase (Hinkkanen & Decker, 1982).

Similar observations to those described here for flavoenzymes were described for enzymes utilizing trace metals as cofactors (Doyle & Arp, 1986; Iuchi & Lin, 1987; Boursier *et al.*, 1988). Micro-organisms rely on the availability of trace metals from the environment, and an adaptive response correlating enzyme biosynthesis with cofactor supply seems reasonable. The riboflavin-dependent expression of the *nic*-regulon genes might

reflect a regulatory response of the bacterial cell which prevents the synthesis of additional flavoenzymes in a situation where flavin supply can satisfy only the demand of enzymes essential for basal metabolic functions. The molecular mechanism(s) involved in this regulation require further investigation.

We thank Professor K. Decker for helpful comments, L. Mauch and S. Grether-Beck for plasmids and Ms. H. Gottschalk for her help with the preparation of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 206).

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