Betaine Stimulation of Vitamin B₁₂ Biosynthesis in *Pseudomonas denitrificans* May Be Mediated by an Increase in Activity of δ -Aminolaevulinic Acid Synthase

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(Received 26 October 1983)

The stimulation by betaine of vitamin B₁₂ and extracellular coproporphyrin production in the industrial species *Pseudomonas denitrificans* GY57/2 was confirmed using a replacement culture technique; biosynthesis of haem was also found to be stimulated. In the absence of betaine, δ -aminolaevulinic acid synthase decreased in specific activity from time zero. In the presence of betaine, the enzyme activity increased markedly after 3 h and then decreased after exhaustion of extracellular betaine. The peak specific activity of δ -aminolaevulinic acid synthase increased in a dose-dependent manner. The enzyme was inhibited by haem. δ -Aminolaevulinic acid dehydratase was present in considerable excess over δ -aminolaevulinic acid synthase under all conditions studied and its specific activity was not affected by the addition of betaine. Our tentative conclusion is that the betaine effect is exerted via some positive effect on δ -aminolaevulinic acid synthase, the initial, rate-limiting and very labile enzyme of porphyrin and corrin biosynthesis.

INTRODUCTION

The methyl donors betaine and choline have been known for more than thirty years to stimulate production of vitamin B₁₂ in some micro-organisms (Ansbacker & Hill, 1949). The specificity of the stimulation for these two compounds was demonstrated by Demain *et al.* (1968) with *Pseudomonas denitrificans*. Demain & White (1971) subsequently found that overproduction of coproporphyrin III as well as vitamin B₁₂ takes place in the presence of betaine. Corrins and porphyrins are products of pathways branching from the common precursor uroporphyrinogen III, which is derived from succinyl-coenzyme A plus glycine via the intermediates δ -aminolaevulinic acid and porphobilinogen. Shemin *et al.* (1956) using ¹⁴C-labelled glycine, δ -aminolaevulinic acid or porphobilinogen demonstrated that the corrin ring of vitamin B₁₂ is constructed in a fashion similar to that leading to the porphyrin ring of haem. White & Demain (1971) then showed that incorporation of [¹⁴C]methyl units of betaine onto the periphery of the corrin nucleus proceeded only indirectly after betaine degradation, by way of methionine. They suggested at that time that the true role of betaine in corrin and porphyrin overproduction was not that of a precursor but was probably of a regulatory nature.

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Abbreviations: ALAS, δ -aminolaevulinic acid synthase (EC 2.3.1.37); ALAD, δ -aminolaevulinic acid dehydratase (EC 4.2.1.24).

METHODS

Cell cultivation. *Pseudomonas denitrificans* GY57/2 was grown in a defined medium containing (per litre): 20 g sucrose, 4 g each of diammonium hydrogen phosphate, sodium glutamate and sodium citrate dihydrate plus trace metals (Lago & Demain, 1969). Culture volumes of 500 ml were used in 2.8-l Fernbach flasks at 28°C with continuous agitation (250 r.p.m., 5 cm orbit) until an optical density equivalent to 5 g cell dry weight l^{-1} was achieved. Cells were centrifuged, washed, and resuspended in a replacement medium containing 10 g sucrose l^{-1} , 2 g diammonium hydrogen phosphate l^{-1} and one-quarter the concentration of metals used in the growth medium. Betaine was added to required concentrations at this time ($t = 0$) along with 25 mg 5,6-dimethylbenzimidazole l^{-1} as a vitamin B₁₂ precursor. Washed cells (100 ml volumes, 5 g cell dry wt l^{-1}) were incubated as above in 500 ml Erlenmeyer flasks. Samples were withdrawn for analyses at zero time and at appropriate intervals thereafter.

Vitamin B₁₂ assay. Vitamin B₁₂ was quantified by an agar-diffusion assay with *Lactobacillus lactis* (Dorner) ATCC 10697 essentially according to Cuthbertson *et al.* (1951), except that samples were applied to penicillin assay discs and placed onto the dry agar surface instead of adding the samples to pre-cut wells. Authentic vitamin B₁₂ standards were applied on separate discs in each plating of samples to serve as an internal control. After incubation overnight at 37°C in a humidified incubator, the diameters of the growth zones surrounding the discs were determined with a Fisher-Lilly reader. Plots of the logarithm of concentration vs zone diameter were linear with standards ranging from 0.1 to 3.2 µg ml^{-1} with 20 µl of standard applied to each disc.

Cultures of *P. denitrificans* were prepared for bioassay of total vitamin B₁₂ by boiling samples containing 2% (w/v) NaNO₂ and 0.1% (w/v) KCN at pH 3.5 in a water bath for 3 min, thus converting the unstable B₁₂ coenzyme to the more stable cyanocobalamin (White & Demain, 1971). Samples were frozen until assays could be performed, which was usually within a few days.

Coproporphyrin determination. Coproporphyrin was determined from the absorbance at 401 nm (molar absorption coefficient = 489000 $l\ mol^{-1}\ cm^{-1}$) of cell-free culture broths acidified to 0.1 M-HCl. The Rimington (1960) correction procedure was used to correct the measured absorbance at 401 nm. Authentic coproporphyrin was used in each determination to derive the constant, *K*, used in making the correction.

When excessive amounts of materials were present which interfered with absorbance measurements, coproporphyrin was extracted into diethyl ether at pH 4.5 (sodium acetate buffer). The ether layer was extracted twice with 3 M-HCl to recover the coproporphyrin. The latter was concentrated by extracting into ether after addition of sufficient saturated sodium acetate to increase the pH to about 4. The ether was evaporated, and the residue dissolved in 0.1 M-HCl.

Haem extraction and analysis. The method used was essentially that of Teale (1959). Wet cell pastes were acidified to 0.1 M-HCl, and extracted 3 times with 3 volumes of methyl ethyl ketone. After evaporation of the solvent, pyridine haemochromogen determinations were done according to Paul *et al.* (1953) using a difference molar absorption coefficient [557 nm (reduced) minus 540 nm (oxidized) = 20700 $l\ mol^{-1}\ cm^{-1}$].

This extraction method was also used to prepare globin from methaemoglobin (Sigma).

Other analyses. Betaine was measured essentially according to Wall *et al.* (1960) as modified by Barak & Tuma (1979). δ -Aminolaevulinic acid synthase (ALAS EC 2.3.1.37) was determined according to Burnham (1970). δ -Aminolaevulinic acid dehydratase (ALAD EC 4.2.1.24) was determined according to Shemin (1970). Enzyme assays were done on crude extracts prepared by washing cells once in a volume of 20 mM-Tris/HCl (pH 7.5) buffer equivalent to the broth volume, resuspending them in an equal volume of the same buffer, sonicating with a Branson Sonifier until a 90% reduction in absorbance was attained, and removing debris by centrifugation. Protein was determined by the Bradford (1976) procedure with bovine serum albumin as standard. Coproporphyrin standards and reagents for enzyme assays were obtained from Sigma.

RESULTS

Overproduction of vitamin B₁₂, coproporphyrin and haem in cells grown in the presence of betaine

Figures 1(a), (b), (c) and (d) illustrate the effects of cell growth in replacement medium containing 0, 5.0, 7.5 and 10.0 g l^{-1} , respectively, of betaine. Quantification of the major products accumulating in the presence of betaine has been normalized with respect to cell mass, and is reported here as nmol per g cell dry weight. When expressed in this manner, net synthesis of products will generate curves with positive slopes, no net synthesis will give horizontal lines, while destruction of products or lack of synthesis during continued cell growth will form curves with negative slopes.

In the absence of betaine (Fig. 1 a), coproporphyrin was not detected extracellularly and slight net decreases were observed in total (cellular and extracellular) vitamin B₁₂ and cellular haem. In the presence of betaine (Fig. 1 b, c, d) net synthesis of all three substances was observed. The specific content of vitamin B₁₂ increased several-fold during the 30 h period in the presence of

Fig. 1. Oversynthesis of coproporphyrin, haem and vitamin B₁₂ in the presence of betaine at (a) 0, (b) 5.0, (c) 7.5 and (d) 10.0 g l⁻¹, respectively. ▲, Cell dry weight; □, B₁₂; △, haem; ■, coproporphyrin; ●, betaine.

all three concentrations of betaine. Coproporphyrin accumulation was maximal with 7.5 g betaine l⁻¹. In all cases, oversynthesis of this porphyrin stopped sharply upon exhaustion of the betaine. By 30 h, the specific content of solvent-extractable haem had increased several-fold, but the kinetics of accumulation were complex. No net accumulation of haem was seen until after about 15 h; before that time some apparent decrease took place.

Effects of growth in the presence of betaine on early enzymes of the porphyrin/corrin biosynthetic pathway

In many organisms, the first enzyme of the haem biosynthetic pathway, ALAS, is known to be both repressed and inhibited by haem (Burnham & Lascelles, 1963; Lascelles & Hatch, 1969; Granick, 1966). In *Neurospora crassa*, the second enzyme of the pathway, ALAD, is known to be repressed by protoporphyrin (Chandrika *et al.*, 1980).

To examine the effects of cell growth in the presence of betaine on the specific activities of

Fig. 2. Increase in ALAS activity in the presence of betaine at (a) 0, (b) 2.0, (c) 4.0 and (d) 6.0 g l⁻¹, respectively. The arrows indicate when betaine was exhausted. ▲, Cell dry weight; □, B₁₂; ■, coproporphyrin; ▽, ALAD; ○, ALAS - globin; ●, ALAS + globin. Globin was added at 1 mg ml⁻¹.

ALAS and ALAD, cells were washed and resuspended in replacement medium containing 0, 2.0, 4.0 and 6.0 g betaine l⁻¹. Samples were removed at intervals and assayed for cell growth, coproporphyrin, vitamin B₁₂, betaine and specific activities of ALAS and ALAD in crude extracts. Although not shown here, the addition of haemin to bacterial extracts prepared by sonication resulted in inhibition of ALAS activity in the *in vitro* assay. Half-maximal inhibition was obtained with 0.3×10^{-6} M-haemin which agrees well with the value of 0.25×10^{-6} M as the K_i for haem inhibition of ALAS from *Rhodopseudomonas spheroides* (Burnham, 1970). The addition of haem-free globin (1 mg protein ml⁻¹) to the assay system either before or after addition of 1×10^{-6} M-haemin prevented inhibition of catalytic activity. Assays were routinely performed with and without the addition of globin.

As shown in Fig. 2(a) (no betaine), ALAS activity declined progressively with time, as did vitamin B₁₂. Extracellular coproporphyrin was again not detectable. ALAD specific activity

Fig. 3. Effects of haemin addition on growth and production of vitamin B₁₂, coproporphyrin and ALAS activity. (a) 6.0 g betaine l⁻¹; (b) 6.0 g betaine l⁻¹ plus 10⁻⁴ M-haemin. Where added, haem-free globin was present at 4 mg ml⁻¹ in the *in vitro* ALAS assay. ▲, Cell dry weight; □, B₁₂; ■, coproporphyrin; ○, ALAS - globin; ●, ALAS + globin.

was in great excess over the ALAS specific activity at all times. In the presence of 2.0, 4.0 and 6.0 g betaine l⁻¹ (Fig. 2*b, c, d*), ALAS specific activity declined during the first 3 h as in the control, although not as markedly; it then rose sharply. Immediately upon exhaustion of betaine, indicated in Fig. 2 by the arrows, ALAS activity fell precipitously and returned to the basal levels seen in the control without betaine. Betaine appeared to have no marked effect on ALAD specific activity. With all three concentrations of betaine used, abrupt concurrent decreases in rates of synthesis both of vitamin B₁₂ and of coproporphyrin occurred within a few hours after betaine exhaustion, i.e. the period during which ALAS specific activities were declining. The maximum accumulation both of vitamin B₁₂ and of coproporphyrin was proportional to the amount of betaine supplied initially, and to the area under the ALAS specific activity curves.

Haem analysis was not done in this experiment. However, other experiments show (e.g. Fig. 1) that synthesis of solvent-extractable haem occurred in response to the addition of betaine. Since some fraction of the total haem synthesized may not be complexed as haemoprotein, it might, therefore, be available to inhibit either the activity or the synthesis of ALAS (Granick, 1966). Evidence for the former possibility is seen in Fig. 2(*b, c, d*). When the ALAS assays were performed with added globin, which would bind traces of free haem, a stimulation of ALAS activity was observed. However, even in this case, ALAS specific activity increased in response to betaine addition and markedly declined upon exhaustion of betaine.

Effects of addition of haemin

In an attempt to determine whether haem exerts a regulatory influence on the synthesis of ALAS, cells were washed and resuspended in replacement medium containing 6.0 g betaine l⁻¹. Two flasks were prepared, and to one was added a sterile solution of haemin to provide 1 × 10⁻⁴ M-haemin. As shown in Fig. 3(*a*), the control without haemin was virtually identical in all measured parameters to the culture illustrated in Fig. 2(*d*), which also contained 6.0 g betaine l⁻¹. Fig. 3(*b*) shows that exogenously added haemin prevented cell lysis and caused a

moderate delay in the overproduction of vitamin B₁₂ and coproporphyrin, although final net accumulations were about the same as in the absence of added haemin. When ALAS was measured in the absence of globin, activities were significantly lower in the haemin-supplemented culture than in the control; this was expected because of haem inhibition of ALAS activity. When globin was added in the *in vitro* assay of control samples, ALAS specific activity reached a maximum by about 9 h and was maintained at this plateau until betaine depletion occurred between 18 and 21 h. When globin was added to the assay samples from the haemin-supplemented culture, no plateau of enzyme specific activity was seen. Rather, specific activity continued to increase until betaine was exhausted. The maximum specific activity of ALAS was 50% higher in the presence of haemin than in its absence. Although this experiment suggests that haemin exerts no repression of ALAS, the possibility exists that haemin cannot enter the cells of *P. denitrificans* and its prevention of lysis and retardation of vitamin B₁₂ and coproporphyrin formation could be due to a direct effect of haemin on the cell membrane.

DISCUSSION

The results obtained essentially confirm the earlier reports by Demain *et al.* (1968) and Demain & White (1971) with regard to oversynthesis of vitamin B₁₂ and of coproporphyrin when betaine is added to cultures of *P. denitrificans*. Newer information has been obtained with respect to kinetics of betaine utilization, synthesis of haem and formation of the initial enzymes of the haem biosynthetic pathway, as well as to the effects of exogenously added haemin.

Daniels (1970) previously reported that betaine supplied at 10 g l⁻¹ to *P. denitrificans* is completely utilized in about 30 h. This has been essentially confirmed (Fig. 1*d*). Moreover, as seen in Fig. 1(*b, c, d*), disappearance of betaine is biphasic; a period of slow initial utilization precedes the development of a more rapid rate of betaine uptake from the medium.

ALAS activity is initially present in moderate amounts in cells cultivated in the growth medium used. Upon transfer to replacement medium without betaine, ALAS specific activity immediately declines to, and remains at, a very low level. If transfer of cells is made into replacement medium containing betaine (Figs 2 and 3), ALAS specific activity drops to about 50% of the initial levels of activity by 3 h. Thereafter, however, activity increases to a maximum plateau level, usually by about 9 h. Upon exhaustion of betaine, activity once again decreases rapidly. Apparently, there is an active turnover of ALAS, as reported in other systems (Sinclair & Granick, 1975), so that the activity present at any time represents the net balance between the rate of *de novo* enzyme formation and the rate of irreversible enzyme inactivation.

Thus, it appears that overproduction of vitamin B₁₂ and porphyrins is limited by the *in vivo* lability of ALAS, and the increase in the specific activity of this rate-limiting enzyme by the addition of betaine is responsible for the stimulatory effect of this compound. It is of considerable interest that drugs which increase porphyrin synthesis in animal cells, e.g. 3,5-dicarbethoxy-1,4-dihydrocollidine and allylisopropylacetamide, act by inducing ALAS (Israels, 1973).

The authors acknowledge the excellent assistance of Wolfgang J. Schneider and his valuable suggestions prior to the studies described here.

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