Mechanism of Ammonia Assimilation in Streptococci

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SUMMARY

Streptococcus sanguis, *S. bovis*, *S. mutans* and *S. salivarius*, and also *Escherichia coli* for comparison, were grown separately in a chemostat under ammonia- and glucose-limitation. Bacterial extracts were assayed for ammonia-assimilating enzymes. For *E. coli* the level of glutamate dehydrogenase decreased and the levels of glutamine synthetase and glutamine (amide) : 2-oxoglutarate aminotransferase (glutamate synthase) increased following a change from glucose- to ammonia-limitation. In contrast, ammonia-limited streptococci contained much higher levels of glutamate dehydrogenase than glucose-limited streptococci, and although a glutamine synthetase was detected in *S. bovis*, glutamate synthase could not be detected in any of the streptococci. Low activity of a NAD-linked alanine dehydrogenase was found in *S. bovis* and *S. sanguis*. These results suggest that the only pathway of ammonia assimilation in streptococci involves glutamate dehydrogenase, irrespective of the nature of the growth limitation. Glutamate dehydrogenase extracted from the different species of *Streptococcus* had similar Michaelis constants and optimum pH values.

INTRODUCTION

Recently, Tempest, Meers & Brown (1970) described a novel pathway for ammonia assimilation in which glutamine (amide) : 2-oxoglutarate aminotransferase (glutamate synthase) and glutamine synthetase (EC. 6. 3. 1. 2) were involved. When *Aerobacter aerogenes* was grown in a chemostat under glucose-limitation, glutamate dehydrogenase (EC. 1. 4. 1. 4) was the primary ammonia-assimilating enzyme. Under ammonia-limitation the level of glutamate dehydrogenase decreased, while the levels of glutamate synthase and glutamine synthetase increased. The importance of glutamate synthase in ammonia assimilation is now well established (Meers, Tempest & Brown, 1970; Nagatani, Shimizu & Valentine, 1971; Elmerich & Aubert, 1971; Dainty, 1972; Meers & Pedersen, 1972; Brown, MacDonald-Brown & Stanley, 1973; Brown, Burn & Johnson, 1973).

The ability of *Streptococcus bovis* to grow in a medium containing ammonium ions as the sole nitrogen source was established by Wolin, Manning & Nelson (1959) but similar simple nutritional requirements for oral streptococci have only recently been demonstrated (Carlsson, 1970, 1971, 1972). *S. bovis* assimilates ammonia by means of a glutamate dehydrogenase (Burchall, Niederman & Wolin, 1964) but lacks glutamine synthetase (Burchall, Reichelt & Wolin, 1964). Also the oral streptococci, *S. mutans* and *S. sanguis*, have glutamate dehydrogenase but lack glutamine synthetase as well as glutamate synthase (Yamada & Carlsson, 1973). However, in neither of these studies were the streptococci grown under ammonia-limitation.

Streptococci are among the predominant bacteria in the oral cavity and it would be of

interest to elucidate further, if and in what way they are able to utilize the low amounts of ammonia (3 to 6 mM) present in saliva (Battistone & Burnett, 1961).

This paper describes experiments in which different strains of streptococci were grown in continuous culture in media of different nutritional complexity and with different growth limitations. One strain of *Escherichia coli* was also included for comparative purposes. Bacterial extracts were prepared and analysed for ammonia-assimilating enzymes.

METHODS

Micro-organisms. The strains used were *S. salivarius* ATCC13419, *S. sanguis* NCTC10904 (strain 804; Carlsson, 1968), *S. mutans* JC2 (Carlsson, 1968) and *S. bovis* ATCC9809. *Escherichia coli* strain Hfr Hayes was kindly supplied by Dr K. Nordström, Department of Microbiology, University of Umeå.

Chemicals. Casamino acids (0230-01), tryptose (0124-01), mitis salivarius agar (0298-01) and yeast extract (0127-01) were obtained from Difco, endo agar (CM 37) was from Oxoid, polypropylene glycol 2025 from BDH, 2-oxoglutaric acid, NADH, NADPH, NADP and ADP were obtained from C. F. Boehringer and Soehne, Mannheim, Germany and L-glutamic acid γ -mono-hydroxamate was from Sigma; all other chemicals were of reagent grade.

Culture media. The media were prepared from stock solutions: (i) Ammonium hydrogen carbonate (1 M) was sterilized by filtration, Millipore membrane filter type G₄, and stored in 10 ml amounts in screw-capped tubes at 4 °C. (ii) The vitamins solution contained (mg/ml): pyridoxine HCl, 1.2; nicotinic acid, 0.23; biotin, 0.0006; thiamine HCl, 0.005; riboflavin, 0.02; calcium pantothenate, 0.12; *p*-aminobenzoic acid, 0.001; and cysteine HCl, 10. The vitamins were dissolved according to Leslie (1961), and the solution was sterilized by filtration and stored in 10 ml amounts in screw-capped tubes at -20 °C. (iii) The salts solution contained (mg/ml): MgSO₄·7H₂O, 40; NaCl, 2; FeSO₄·7H₂O, 2; and MnSO₄·4H₂O, 2. The salts were dissolved according to Williams (1955) and sterilized by filtration. The solution was stored in 5 ml amounts in screw-capped tubes at room temperature. (iv) A glucose solution (20 %, w/v) was autoclaved and stored in 50 ml amounts in screw-capped bottles or in 10 ml amounts in screw-capped tubes at room temperature. (v) Potassium dihydrogen orthophosphate (1 M) solution was autoclaved and stored in 10 ml amounts in screw-capped tubes at room temperature. (vi) TYC solution contained 10 g tryptose, 5 g yeast extract and 2.5 g Casamino acids in 900 ml water, and was autoclaved and stored at room temperature in screw-capped bottles.

All media contained 10 ml vitamins solution, 5 ml salts solution and 10 ml 1 M-potassium phosphate, in a final volume of 1 l. The glucose-limited defined medium has a concentration of 33 mM-glucose and 20 mM-ammonium hydrogen carbonate, and the ammonia-limited defined medium 55 mM-glucose and 5 mM-ammonium hydrogen carbonate. The glucose-limited complex medium contained 33 mM-glucose and 900 ml TYC solution. The nitrogen-limited complex medium had 55 mM-glucose and 75 ml of TYC solution. The concentration of the glucose had to be increased to 66 mM when *Streptococcus bovis* was grown in the nitrogen-limited medium, to maintain a surplus of glucose.

Culture conditions. Two 1 l fermenters (FG-500: Biotec AB, Bromma, Sweden) with stirring, pH and temperature controls were used (Carlsson & Elander, 1973). The media were supplied by peristaltic pumps (LP 600, Biotec AB; 12000 Varioperpex, LKB Produkter AB, Bromma, Sweden). For anaerobic conditions a mixture of 95 % CO₂ and 5 % H₂, sterilized by filtration, was provided (flow rate 50 ml/min) through the main top

joint of the fermenter. Traces of oxygen in the gas were removed by a catalytic purifier (Deoxo D5/50; Engelhard Industries Ltd, Cinderford, Gloucester). For anaerobic conditions, air, sterilized by filtration, was provided through the gas capillary tube and bubbled through the medium (flow rate 200 ml/min). Flow rates of the gases were controlled by flow meters (1100; GEC-Elliott Process Instruments Ltd). Foaming was controlled by the addition of 1 ml polypropylene glycol/l medium. The overflow from the fermenters was collected with a fraction collector (Minirac, LKB Produkter) provided with a freezing bath kept at -35°C (KB-300; Fryka-Kältetechnik, Holgenburg, Germany).

The working volumes of the fermenters were 300 and 175 ml respectively. All strains were grown at a dilution rate (D) of 0.125 h^{-1} . When *Streptococcus sanguis* is grown at that rate, glucose is efficiently utilized and there is an optimal yield of bacteria in complex as well as in defined media (Carlsson & Elander, 1973). The temperature was kept at 37°C and pH at 7.0. At the start of each experiment the fermenter was inoculated with 10 ml of an 18 h culture grown in the complex medium. The culture was considered to be in steady state after input of medium corresponding to 12 working volumes of the fermenter. The turbidity of the culture was measured after each input of medium corresponding to three working volumes of the fermenter, when the purity of the culture was also checked by culturing aerobically and anaerobically on blood agar and mitis salivarius agar or endo agar plates.

Preparation of bacterial extracts. The bacteria in 100 to 200 ml of culture were harvested by centrifuging at 4°C , washed twice with 0.1 M -potassium phosphate buffer (pH 7.0) and re-suspended in 3 ml of the same buffer. Glass beads (1.5 ml, 0.10 to 0.11 mm ; B. Braun, Melsungen, Germany), were added to the suspension and the bacteria disintegrated in a homogenizer (Type MSK, B. Braun) for 3 min under carbon dioxide cooling. The mixture was centrifuged at $40000g$ for 30 min at 4°C and the supernatant fluid was used as crude bacterial extract.

Enzyme assays. Each enzyme was assayed in three independent samples from each steady-state condition and the results quoted represent the mean values. For the glutamate dehydrogenase determinations, the coefficient of variation was from 0.08 to 0.10 for samples obtained from the chemically defined medium and 0.09 to 0.13 for samples from the complex medium.

Glutamate dehydrogenase (EC. 1.4.1.4), alanine dehydrogenase, and glutamine (amide): 2-oxoglutarate aminotransferase activities were estimated from the decrease in extinction at 340 nm due to the oxidation of the nicotinamide nucleotide at 25°C using a double-beam spectrophotometer (DB-G; Beckman Instruments Ltd) with recorder. The volume of the reaction mixture (Yamada & Carlsson, 1973) for assay of each enzyme was 3 ml. For glutamate dehydrogenase, $200\text{ }\mu\text{mol}$ of potassium phosphate buffer (pH 7.6), $100\text{ }\mu\text{mol}$ of NH_4Cl , $0.24\text{ }\mu\text{mol}$ of NADPH, and $15\text{ }\mu\text{mol}$ of 2-oxoglutarate and crude bacterial extract were used. The normal order of addition of substrates was NH_4Cl , NADPH, enzyme extract and 2-oxoglutarate. When determining the Michaelis constant for ammonia the order of addition was NADPH, enzyme extract, 2-oxoglutarate and NH_4Cl . For determining the optimum pH for activity of the enzyme a universal buffer (McKenzie, 1969) was used and the pH of the reaction mixture was checked after each determination. For alanine dehydrogenase $200\text{ }\mu\text{mol}$ of potassium phosphate buffer (pH 7.0), $0.24\text{ }\mu\text{mol}$ of NADH or NADPH $100\text{ }\mu\text{mol}$ of NH_4Cl , and $10\text{ }\mu\text{mol}$ of sodium pyruvate and crude bacterial extract were used. For glutamine (amide): 2-oxoglutarate aminotransferase, $300\text{ }\mu\text{mol}$ of potassium phosphate buffer (pH 7.6), $15\text{ }\mu\text{mol}$ of 2-oxoglutarate, $0.24\text{ }\mu\text{mol}$ of NADPH or NADH, and $15\text{ }\mu\text{mol}$ of L-glutamine and crude bacterial extract were used.

Aspartase and glutaminase activities were estimated by determining the amount of ammonia liberated from aspartate and glutamine by crude bacterial extracts (Halpern &

Umbarger, 1970). The reaction mixture for aspartase determination contained 200 μmol of potassium phosphate buffer (pH 7.6) and 60 μmol of sodium L-aspartate and crude bacterial extract, in a total of 1.5 ml. The reaction mixture for glutaminase determination contained 50 μmol of potassium phosphate buffer (pH 7.6) and 30 μmol of L-glutamine and crude bacterial extract, in a total of 0.5 ml. In both assays the reaction mixture was incubated at 37 °C for various time intervals between 0 and 60 min, when the reaction was stopped by the addition of 3.75 ml of 0.14 M-phenol.

Glutamine synthetase (EC. 6.3.1.2) was assayed by measuring γ -glutamyl-hydroxamate formation (Woolfolk, Shapiro & Stadtman, 1966). The reaction mixture contained in 2 ml: 50 μmol of imidazole buffer (pH 7.0), 12 μmol of hydroxylammonium chloride, 8 μmol of ADP, 10 μmol of MnCl_2 , 40 μmol of sodium arsenate and 60 μmol of L-glutamine and crude bacterial extract. The reaction mixtures were incubated at 37 °C for 60 min, when the reaction was stopped and the colour developed by the addition of 0.5 ml of a solution containing equal volumes of 24% (w/v) trichloroacetic acid, 6 N-HCl and 10% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.02 N-HCl. After centrifuging, the extinction at 540 nm was measured using authentic γ -glutamyl-hydroxamate as a standard.

Units of glutamate dehydrogenase, alanine dehydrogenase and glutamate synthase are defined as μmol of NADH or NADPH oxidized/min, glutaminase and aspartase as μmol of ammonia liberated/min, and glutamine synthase as μmol of hydroxamate formed/min.

Analytical methods. Protein was determined with the Folin phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951), ammonia with the nitroprusside-alkaline hypochlorite reagent (Chaykin, 1969) and glucose with the glucose oxidase method (Glucostat®, Worthington Biochemical Corp.) Dry weight was determined as described by Carlsson (1971).

RESULTS

Ammonia-assimilating enzymes detected in *Escherichia coli* are shown in Table 1. For *E. coli* grown in the chemically defined medium, a change from glucose- to ammonia-limitation caused a decrease in the level of glutamate dehydrogenase and an increase in the levels of glutamate synthase and glutamine synthetase; these changes were also accompanied by a decrease in the amount of glutaminase. When *E. coli* was grown under nitrogen-limitation in the complex medium, the level of glutamate dehydrogenase was high and the level of glutamate synthase low compared with those found in the ammonia-limited defined medium. When grown under glucose-limitation, the level of glutamate dehydrogenase was lower in the complex medium than in the defined medium. The levels of aspartase were higher in the two complex media than in the chemically defined media.

Table 2 shows the levels of ammonia-assimilating enzymes detected in the four strains of streptococci. The levels of glutamate dehydrogenase were greatly increased following a change from glucose-limitation to ammonia- or nitrogen-limitation. Activity of glutamate synthase, glutaminase or aspartase could not be detected in any streptococcal extracts. *S. bovis* was the only streptococcus examined which had a readily detectable glutamine synthetase, and a change from glucose-limitation to ammonia- or nitrogen-limitation increased the level of this enzyme. Low activity of NAD-linked alanine dehydrogenase was found in *S. sanguis* and *S. bovis*.

The K_m for ammonia of the glutamate dehydrogenase of *Escherichia coli* was higher than for any of the streptococcal enzymes (Table 3). The streptococcal glutamate dehydrogenases all had similar K_m values and all showed maximum activity between pH 7.6 and 7.9. The optimum pH range for activity of the glutamate dehydrogenase from *E. coli* was 8.1 and 8.4.

Table 1. *The distribution of possible ammonia-assimilating enzymes in Escherichia coli grown in different media under aerobic conditions*

E. coli was grown in a chemostat ($D = 0.125 \text{ h}^{-1}$, 37°C , pH 7.0) in media containing growth-limiting concentrations of the specified nutrients. The defined medium contained glucose as the energy source and ammonia as the nitrogen source. The complex medium contained glucose as the energy source and TYC as the nitrogen source.

Medium	Growth limiting factor	Enzyme activity (units/mg protein)					
		Glutamate dehydrogenase	Glutamine synthase	Alanine dehydrogenase	Glutamate synthase	Glutaminase	Aspartase
Defined	Glucose	1.0	0.05	0.02	0.02	0.02	0.007
Defined	Ammonia	0.1	0.2	0.05	0.10	0.005	0.005
Complex	Glucose	0.04	0.02	0.03	0.01	0.03	0.03
Complex	Nitrogen	0.7	0.25	0.04	0.02	0.008	0.01

Table 2. *The distribution of possible ammonia-assimilating enzymes in streptococci grown in different media under anaerobic conditions*

The organisms were grown in a chemostat ($D = 0.125 \text{ h}^{-1}$, 37°C , pH 7.0) in media containing growth-limiting concentrations of the specified nutrients. See Table 1 for a description of the media.

Organism	Medium	Growth limiting factor	Enzyme activity (units/mg of protein)		
			Glutamate dehydrogenase	Glutamine synthase	Alanine dehydrogenase
<i>Streptococcus mutans</i> JC2	Defined	Glucose	1.1	< 0.005	< 0.005
	Defined	Ammonia	6.3	< 0.005	< 0.005
	Complex	Glucose	0.5	< 0.005	N.D.
	Complex	Nitrogen	11.2	< 0.005	< 0.005
<i>Streptococcus salivarius</i> ATCC13419	Defined	Glucose	1.2	< 0.005	< 0.005
	Defined	Ammonia	6.5	< 0.005	< 0.005
	Complex	Glucose	0.5	< 0.005	< 0.005
	Complex	Nitrogen	15.8	< 0.005	< 0.005
<i>Streptococcus sanguis</i> NCTC10904	Defined	Glucose	3.2	< 0.005	0.10
	Defined	Ammonia	13.8	< 0.005	0.25
	Complex	Glucose	2.5	< 0.005	N.D.
	Complex	Nitrogen	11.9	< 0.005	0.20
<i>Streptococcus bovis</i> ATCC9809	Defined	Glucose	1.6	0.04	0.09
	Defined	Ammonia	8.3	0.20	0.06
	Complex	Glucose	0.05	0.03	N.D.
	Complex	Nitrogen	4.0	0.40	0.09

N.D. = Not determined because of high levels of NADH oxidase.

The steady-state concentration of ammonia remaining in the ammonia-limited defined medium was $50 \mu\text{M}$ after the growth of *Streptococcus mutans* and less than $10 \mu\text{M}$ after growth of all the other streptococci. The concentration of glucose remaining in the glucose-limited defined medium varied between 9 and $13 \mu\text{M}$.

The yield of *Escherichia coli* in the ammonia-limited defined medium was $0.21 \text{ mg dry wt/ml}$. The yield of the four strains of streptococci in the ammonia-limited defined medium varied between 0.22 and $0.38 \text{ mg dry wt/ml}$.

Table 3. *Apparent Michaelis constants (K_m) for ammonia of different bacterial glutamate dehydrogenases*

The streptococcal enzyme extracts were prepared from bacteria grown in the ammonia-limited defined medium, whereas the *Escherichia coli* extracts were prepared from bacteria grown in the glucose-limited defined medium. The bacterial extracts were dialysed overnight against 0.1 M-potassium phosphate buffer (pH 7.0).

Organism	Apparent K_m for ammonia (mM)	
	Mean	SEM
<i>Escherichia coli</i>	4.9	± 0.2
<i>Streptococcus bovis</i>	2.7	± 0.4
<i>S. sanguis</i>	1.9	± 0.2
<i>S. salivarius</i>	2.2	± 0.1
<i>S. mutans</i>	2.9	± 0.2

DISCUSSION

The mechanisms of ammonia assimilation in *Escherichia coli* appear to be similar to those described for *Aerobacter aerogenes* (Meers *et al.* 1970). Glutamate dehydrogenase assimilates ammonia when the organisms are grown under glucose-limitation and glutamine synthetase and glutamate synthase when the organisms are grown under ammonia-limitation.

The glutamate dehydrogenase of streptococci appears to be regulated in a way not previously described for bacteria. A change from glucose-limitation to ammonia-limitation produced a large increase in the level of glutamate dehydrogenase. Glutamate synthase was not detected in any of the streptococci and glutamine synthetase, a pre-requisite for the glutamate synthase pathway, was only detected in *S. bovis*. It would therefore seem that glutamate dehydrogenase is the only enzyme for ammonia assimilation present in streptococci, irrespective of the nature of the growth limitation.

Klebsiella pneumoniae mutants, which did not possess glutamate synthase, failed to grow in media with low concentrations of ammonia as the main nitrogen source (Nagatani *et al.* 1971). However, some yeasts do not possess the glutamate synthase pathway but are able to grow in media containing 2 mM-ammonia as the nitrogen source (Brown & Stanley, 1972). In the present study the streptococci could be kept in continuous culture in a defined medium containing 5 mM-ammonia ($D = 0.125 \text{ h}^{-1}$), and *Streptococcus mutans*, the only strain tested, continued to grow even if the ammonia concentration was decreased to 2 mM ($D = 0.125 \text{ h}^{-1}$).

Meers *et al.* (1970) suggested that under conditions of ammonia-limitation the relatively high K_m for ammonia of bacterial glutamate dehydrogenase would make this enzyme functionally inadequate, and that glutamine synthetase, by virtue of its lower K_m for ammonia, would be better suited to assimilate the ammonia. Although the streptococcal glutamate dehydrogenases had a somewhat lower K_m for ammonia than that of *Escherichia coli*, the differences were not large enough to explain the difference in mechanisms of ammonia assimilation. Results of glutamate dehydrogenase K_m determinations for ammonia are known to vary with the buffer used (Fahien & Strmecki, 1969) and even with the order of addition of substrates (Coulton & Kapoor, 1973), and it is possible that the *in vivo* K_m for ammonia of the streptococcal glutamate dehydrogenase enzymes is much lower than values obtained during *in vitro* determinations. Coupling of glutamate dehydrogenase with an aminotransferase can lower its K_m for ammonia (Fahien & Smith, 1969; Fahien, Lin-Yu, Smith & Happy, 1971). For this coupling to occur high levels of both glutamate dehydrogenase and aminotransferase are required. High levels of glutamate dehydrogenase were

found in the present study, and large amounts of a glutamate-oxalacetate aminotransferase have been detected recently in the same strain of *S. sanguis* as used in this study and in strain PK1 of *S. mutans* (T. Yamada, personal communication). However, it is possible that the large amounts of the glutamate dehydrogenase produced by the streptococci are in themselves enough for efficient assimilation of even low concentrations of ammonia. That this system was efficient was proved by the very low concentrations of ammonia found in the ammonia-limited medium after growth of the streptococci. All the organisms grown in the ammonia-limited medium had a similar yield (in terms of dry weight) which suggests that the streptococcal glutamate dehydrogenase pathway was as efficient as the glutamate synthase pathway of *E. coli*.

Considering the availability of ammonia, amino acids and other nitrogen-containing compounds in the mouth (Kirch, Kesel, O'Donnell & Wach, 1947; Ludwick & Fosdick, 1955; Battistone & Burnett, 1961; Critchley, 1969; Golub, Borden & Kleinberg, 1971) and the efficiency with which streptococci can assimilate low concentrations of ammonia, it would seem unlikely that streptococci growing in the mouth were nitrogen-limited.

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