FEMS Microbiology Letters 36 (1986) 269–273
Published by Elsevier

FEM 02542

Proline reduction by *Clostridium sporogenes* is coupled to vectorial proton ejection

(*Clostridium sporogenes*; Stickland reaction; proline reductase; proton pump)

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Received 5 June 1986
Accepted 6 June 1986

1. SUMMARY

The reduction of proline by *Clostridium sporogenes* NCIB8053 is coupled to transmembrane proton translocation in an uncoupler-sensitive fashion (and might therefore conserve free energy). This finding serves to explain the increase in the growth yield of this organism when proline is added to a defined growth medium containing glucose as the catabolic substrate.

2. INTRODUCTION

It has been known since the work of Stickland [1] that *C. sporogenes* can obtain the energy it requires for growth from the coupled oxidation and reduction of pairs of amino acids, a reaction (pathway) which now bears his name [2–4]. Early work showed that both glycine [5] and proline [6] could serve as acceptor amino acids, and Stadtman and her colleagues have demonstrated that the reduction (reductive deamination) of glycine to acetate and ammonia by a soluble glycine reductase complex from *Clostridium sticklandii* is coupled to ATP synthesis [7], apparently via substrate-level phosphorylation. The situation with proline reductase is less than clear, however [3], for whilst proline reduction (to δ-amino valerate) by a proline reductase complex is easily demonstrated in vitro, coupled phosphorylation has not been observed to date.

One way to approach this problem is at the physiological level, and our own studies (R.W.L., J.G.M. and D.B.K., in preparation) have indicated that the maximum growth yield of *C. sporogenes* in glucose-limited chemostat culture is significantly increased by the addition of proline (as electron acceptor) to a defined minimal medium, suggesting that the reduction of proline is indeed coupled to ATP synthesis (or at least to free energy conservation).

It is known [2,3,8–12] that the intermediary electron donor for proline reduction in vivo is NADH, which serves to transfer reducing equivalents to the proline reductase enzyme by means of a flavoprotein and an iron-containing protein constituting part of the proline reductase complex, whilst in vitro a variety of di-thio compounds can serve as direct electron donors to the proline reductase enzyme [3]. Since the proline reductase is strongly membrane-associated [13], it occurred to

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us that the inability to demonstrate phosphorylation coupled to proline reduction in vitro might be due to the fact that the reduction of proline conserves free energy by means of a proton gradient. In this vein, Bader and Simon [14] have shown that the reduction of cinnamate by a membrane-associated enoate reductase in *C. sporogenes* is also coupled to phosphorylation in a reaction that is inhibited by the protonophore carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone and the energy transfer inhibitor dicyclohexyl carbodiimide, although the coupling of cinnamate reduction to proton translocation was not studied. The present paper demonstrates for the first time that the reduction of proline by intact cells of *C. sporogenes* is coupled to vectorial proton ejection.

3. MATERIALS AND METHODS

3.1. Source and maintenance of organism

C. sporogenes NCIB8053 was obtained from the National Collection of Industrial and Marine Bacteria as a freeze-dried culture. Cultures were resuscitated on Reinforced Clostridial Medium (RCM Lab M) in both broths and plates (containing 1.5% (w/v) agar). The organisms were freeze-dried in horse serum for long-term storage and were regularly subcultured on RCM at 37°C. Viable cultures could be maintained for over 2 months when stored at 4°C.

3.2. Media

The medium was based upon a low-phosphate basal medium (LPBM) of the following composition (g/l): KH_2PO_4, 2.0; K_2HPO_4, 2.0; $MgCl_2 \cdot 6H_2O$, 0.2; NH_4SO_4, 5.0; 10% $NaHCO_3$, 25 ml added after sterilisation; 0.01% w/v resazurin solution, 1 ml; vitamin solution, 1 ml added after sterilisation; trace element solution, 10 ml. The trace element solution contained (g/l): nitrilo triacetic acid, 12.8; $FeSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 2H_2O$, 0.17; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.01; H_3BO_4, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.01; NaCl, 1.0; $NaSeO_3$, 0.017; $NiSO_4 \cdot 6H_2O$, 0.026; $NaWO_4 \cdot 2H_2O$, 0.1. The vitamin solution, which was sterilized by autoclaving (15 psi, 121°C, 15 min), contained the following (mg/l): thiamine HCl, 50; biotin, 5; PABA, 5; nicotinic acid, 500. The high nicotinic acid concentration maintained the pH at a low value and served to stabilise the thiamine to autoclaving. The medium pH was adjusted to 6.8 prior to autoclaving; following the addition of the bicarbonate solution the pH was approx. 7.25. After all supplemental medium components were added, 5% (w/v) cysteine HCl (pH 6) was added until reduction was effected (as judged by the discolouration of the resazurin); this required about 5–10 ml per l medium.

Essential amino acid/fatty acid (EAA/FA) medium was LPBM supplemented with 3 mM glycine, 2 mM arginine, 1 mM each of histidine, methionine, phenylalanine, tryptophan, 2-methyl propionic acid, 3-methyl butyric acid and 2-methyl butyric acid, and 0.125 mM tyrosine. L-Amino acids (except for 50 mM tryptophan, which was filter-sterilised) were made up as a concentrated solution and sterilised by autoclaving. Neutralised fatty acids were made up as 40 mM solutions and sterilised by autoclaving, whilst yeast extract and tryptone were 10% w/v solutions. L-proline and glucose solutions (20% w/v) were sterilised separately and added to concentrations of 20 mM and 45 mM respectively.

3.3. Cultivation of organisms

Organisms were grown in pressure tubes (Bellco, Vineland, NJ) containing 10 ml medium. The medium (LPBM) was dispensed anaerobically into the pressure tubes with a headspace of 'oxygen-free' (< 4 ppm) nitrogen, the tubes were autoclaved and the other components added aseptically. Prereduced tubes were inoculated with 0.2 ml of culture via a syringe and incubated at 37°C without shaking. Larger volumes were cultured in 2-l Buchner flasks sealed with a rubber bung and fitted with a sample port, bladder and gas filters so as to allow flushing and evacuation of the flask headspace. The flasks, containing 1.5 l medium, were autoclaved (15 psi, 15 min) and after cooling, the sterile supplements necessary for EAA/FA medium, including glucose and proline, were added and the headspace was filled with N_2/CO_2 (95 : 5). Finally, the medium was prereduced with cysteine and inoculated with 5 ml of a 15 h

pressure tube culture. Cultures were grown to mid-to-late exponential phase (0.4 g dry wt./l, approx. 12–14 h after inoculation) at 37°C and were harvested and washed twice with an anaerobic solution of 150 mM KCl containing 5 mM $MgSO_4$. The organisms were resuspended at a concentration of approx. 20 mg dry weight/ml in this buffer and stored anaerobically on ice for up to 8 h.

3.4. Estimation of growth

Growth was estimated by the measurement of the absorbance at 680 nm of a culture sample, 1 absorbance unit corresponding to 320 mg dry wt./l when measurements were made in a cuvette with a path-length of 10 mm. A detailed analysis of the growth energetics, physiology and fermentation products of this organism in this medium will be given elsewhere.

3.5. Measurement of vectorial proton translocation

The reaction mixture was placed in a pH-measuring cell described previously [15–17] and contained, in a final volume of 7.5 ml: 150 mM KCl, 5 mM $MgSO_4$, 0.15 mM K^+-Mops, 480 μg carbonic anhydrase and 10 mg dry weight of the cell suspension described above. Under these conditions, the majority of the buffering power of the system is due to the cells themselves. Other additions are given in the legends to the figures. The temperature was maintained at 37°C by means of a circulating water jacket and the pH of the suspension was held at a value of 6.5 by the addition of small volumes of anaerobic HCl or KOH. The voltage difference between a Russell pH electrode and an Ag/AgCl (3 M KCl) reference electrode was amplified by an ISEAMP 100B (Dulas Engineering, Machynlleth, Wales) and led to a potentiometric chart recorder. The reaction vessel and all solutions were maintained under an atmosphere of oxygen-free nitrogen; pH changes were converted to ng ion H^+ by calibration with anaerobic HCl and KOH in the usual way [15,17,18].

3.6. Chemicals and biochemicals

These were from previously disclosed sources [19,20]. Tetrachloro salicylanilide (TCS) was a generous gift from Professor W.A. Hamilton. Water was singly distilled in an all-glass apparatus.

4. RESULTS AND DISCUSSION

Fig. 1 shows a typical trace in which the pH of a weakly-buffered suspension of *C. sporogenes* is displayed just prior to and following the addition of a pulse of D-proline. In the absence of proline there was a small, continuing rate of (scalar) acid production, due to the generation of acidic end-products (predominantly acetate; data not shown) from endogenous reserves. This background acid production was not abolished by the addition of a protonophore (TCS, 5 μM), was initially somewhat stimulated by the addition of sodium arsenate (0.1–1 mM) and was much enhanced when the pH was made more alkaline than that used in the present work (data not shown). When the suspension was allowed to equilibrate for at least 10 min prior to the proline pulse, however, this background acid production did not proscribe the as-

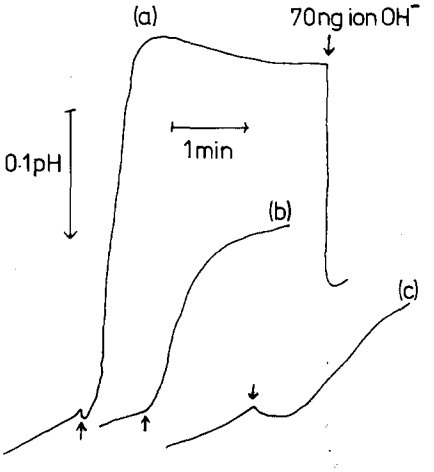

Fig. 1. Proton ejection coupled to the reduction of proline by *C. sporogenes*. The reaction mixture was as given in the experimental section. (a) At the first arrow, D-proline (100 nmol) was added, whilst the second arrow marks the addition of a calibrating pulse of KOH (71 ng ion OH^-). (b), As (a), except that 50 nmol D-proline was added at the arrow. (c), As (b) except that 5 μM tetrachlorosalicylanilide was added 10 min prior to the addition (at the arrow) of 50 nmol D-proline.

sessment of proton translocation linked to the reduction of proline, provided, of course, that an exogenous source of carbon and electrons was not present.

Fig. 1 (a) shows the change in pH which was elicited when a pulse of proline (100 nmol) was added to the suspension; following a brief period of alkalinisation, a rapid and marked acidification of the phase external to the organisms was observed. That the action of D-proline was not simply catalytic is shown in Fig. 1 (b), where it may be observed that the addition of 50 nmol D-proline led to a net acidification approximately half that in Fig. 1 (a). When tetrachlorosalicylanilide, a protonophore, was present at a concentration of 5 μM (Fig. 1 (c)), the proline-induced acidification was abolished and was replaced by a small, net alkalinisation. Thus some component of the electron transport chain of which proline is the terminal electron acceptor is protonmotive. Whilst D-proline is the substrate of the (purified) proline reductase enzyme [3], traces similar to those in Fig. 1 were elicited by the addition of L-proline, a result to be expected in view of the active proline racemase possessed by this organism [3].

Nothing whatever is known of the ion uptake systems possessed by this organism, nor of the site of proline reduction (i.e., whether it is on the inner or outer face of the cytoplasmic membrane). For this reason it is premature to enquire into the source of the alkalinisation preceding the proline-induced acidification, although it could be explained by a proline-H^+ symport or by the combined activity of a cation-proline symport with a cation-H^+ antiport. Amongst the clostridia, it is known that *Clostridium pasteurianum* possesses an active K^+ uniporter [19], which would serve to explain why the addition of an ionophore such as valinomycin or a 'permeant' ion such as thiocyanate was not required to observe proton motive activity. Indeed, neither treatment increased the net observable stoichiometry of proline-induced proton translocation (data not shown).

When the size of the proline pulse was varied in a systematic fashion, the net extent of H^+ translocation varied in proportion (Fig. 2) over a wide range, a result in line with previous findings from

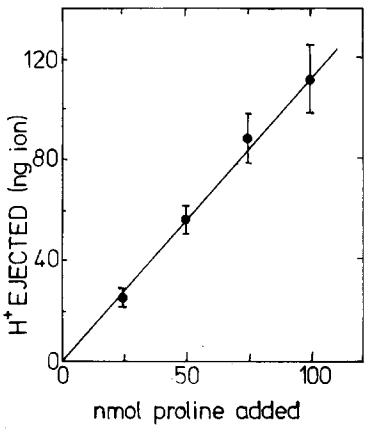

Fig. 2. Effect of the size of the proline pulse on the extent of proton ejection by *C. sporogenes*. Measurements were made as described in the legend to Fig. 1, except that the size of the proline pulse was varied as indicated. The stoichiometry of the reaction (i.e., the $\rightarrow H^+$/proline ratio) is equal to the slope of this graph. Values are given as the mean $\pm$ standard deviation ($N = 4$–7).

this laboratory [15,17,21] which have indicated that provided that adequate 'natural' uniports are present the $\rightarrow H^+/e^-$ ratio of protonmotive systems is independent, over a wide range, of the size of the pulse of electron acceptor. The net observable stoichiometry of proline-induced H^+ ejection (i.e., the $\rightarrow H^+$/proline ratio) may be seen (Fig. 2) to be slightly in excess of 1.

It would be of interest to be able to determine the exact site of protonmotive activity in this system. However, attempts (based on the use of dithiothreitol as an exogenous electron donor [3]) to test the hypothesis that the proline reductase enzyme per se is the protonmotive part of the respiratory chain were unsuccessful, due to our inability fully to inhibit the endogenous source of reducing equivalents. Such studies will therefore have to await the production of a suitably purified proline reductase reconstituted into proteoliposomes.

In conclusion, we have shown here for the first time that the reduction of proline, a constituent part of the Stickland reaction exploited by many proteolytic clostridia, is coupled to vectorial, transmembrane proton translocation. The extent

to which this is true for other Stickland acceptors remains to be assessed.

ACKNOWLEDGEMENTS

We thank the Biotechnology Directorate of the Science and Engineering Research Council, U.K. for financial support.

REFERENCES

[1] Stickland, L.H. (1934) Biochem. J. 28, 1746–1759.
[2] Nisman, B. (1954) Bacteriol. Rev. 18, 16–42.
[3] Seto, B. (1980) in Diversity of Bacterial Respiratory Chains (Knowles, C.J., Ed.), Vol. II, pp. 49–64. CRC Press, Boca Raton, FL.
[4] Barker, H.A. (1981) Annu. Rev. Biochem. 50, 23–40.
[5] Stickland, L.H. (1935) Biochem. J. 29, 896–898.
[6] Stickland, L.H. (1935) Biochem. J. 29, 288–290.
[7] Stadtman, T.C., Elliot, P. and Tiemann, L. (1958) J. Biol. Chem. 231, 961–973.
[8] Mamelak, R. and Quastel, J.H. (1953) Biochim. Biophys. Acta 12, 103–120.
[9] Stadtman, T.C. and Elliot, P. (1957) J. Biol. Chem. 228, 983–997.
[10] Stadtman, T.C. (1962) Arch. Biochem. Biophys. 99, 36–44.
[11] Stadtman, T.C. (1966) Arch. Biochem. Biophys. 113, 9–19.
[12] Schwartz, A.C. and Muller, W. (1979) Arch. Microbiol. 123, 203–208.
[13] Seto, B. and Stadtman, T.C. (1976) J. Biol. Chem. 251, 2435–2439.
[14] Bader, J. and Simon, H. (1983) FEMS Microbiol. Lett. 20, 171–175.
[15] Kell, D.B. and Hitchens, G.D. (1982) Faraday Discuss. Chem. Soc. 74, 377–388.
[16] Hitchens, G.D. and Kell, D.B. (1982) Biochem. J. 206, 351–357.
[17] Hitchens, G.D. and Kell, D.B. (1984) Biochim. Biophys. Acta 768, 222–232.
[18] Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 309–323.
[19] Clarke, D.J., Morley, C.D., Kell, D.B. and Morris, J.G. (1982) Eur. J. Biochem. 127, 105–116.
[20] Booth, I.R. and Morris, J.G. (1975) FEBS Lett. 59, 153–157.
[21] Kell, D.B. and Hitchens, G.D. (1983) in Coherent Excitation in Biological Systems (Fröhlich, H. and Kremer, F., Eds.) pp. 178–198. Springer, Heidelberg.