THE MEMBRANE POTENTIAL AS THE DRIVING FORCE FOR THE ACCUMULATION OF LYSINE BY *STAPHYLOCOCCUS AUREUS*

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1. Introduction

One of the most significant attributes of the chemiosmotic hypothesis [1] is that it relates directly not only to oxidative phosphorylation, but to all of the many energetic and control functions associated with cellular membranes. This feature is particularly relevant in the study of energy coupling mechanisms in bacteria with their single membranous structure. In the field of transport, one would predict that both the major components of the protonmotive force, membrane potential and pH gradient, might be capable of driving the transport of ions and nutrients across membranes against their prevailing electrochemical gradients. A flux of cations might therefore be driven by the membrane potential (inside negative), while anion transport might respond to the pH gradient (inside alkaline) through the action of a proton-symport; such a co-transport with protons would give a positively charged entity with, for example, the neutral sugars, which could then respond to both the membrane potential and the pH gradient [2, 3]. We have set out to test this prediction with respect to the transport of the basic, neutral and acidic amino acids into the intracellular pool of *Staphylococcus aureus*. This paper deals specifically with our findings with the most experimentally accessible case, that of lysine, which, at neutral pH, exists predominantly as the positively charged species. Metabolically depleted cells, possessing relatively high proton and potassium permeabilities, maintain protons near Donnan equilibrium. In such cells there is a membrane potential which is largely an ionic diffusion potential; this may be varied by ionic manipulation, for example, by varying the extracellular potassium concentration in the presence of valinomycin. We have been able to demonstrate that the intracellular accumulation of lysine may represent a Donnan equilibrium driven by this membrane potential, lysine effectively exchanging with intracellular potassium.

2. Materials and methods

2.1. Preparation of cell suspensions

S. aureus was grown for 18 hr at 30°, with shaking, in 100 ml volumes of a medium containing, (g/ℓ); tryptone, 10.0; Lab Lemco, 5.0; yeast extract, 1.0; $Na_2HPO_4 \cdot 12\,H_2O$, 5.0; glucose, 20.0; HCl to pH 6.5. Cells were harvested at room temp. by centrifugation (3,100 *g* 7 min), washed once in 33 mM sodium phosphate buffer, pH 7.0, (70 ml), and resuspended in that buffer (50 ml). To reduce the endogenous reserves of amino acids, the cells were incubated, with shaking, for 3 hr at 37°, after which the internal amino acid levels had attained a steady state. The cells were harvested as above, washed with distilled water (35 ml), and resuspended to approx. 2 ml in 0.25 mM N-Tris (hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), 0.1 mM Tris, pH 7.0. 1.0 ml of this suspension (stored at 0–4°) was used for each experimental incubation.

2.2. Uptake studies

In all experiments, endogenous-reduced cells were used at a final conc. of approx. 1 mg dry bacterial weight/ml. 42 ml reaction volumes were routinely contained in a glass vessel surrounded by a water jacket which allowed pH and pO_2 to be monitored while the

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suspension was both stirred and bubbled with nitrogen. Details of suspension media and concentrations of additives accompany the relevant figures. The antibiotics were added as 20 μl aliquots in acetone, KCl as aliquots of anaerobic 4 M KCl, and NaOH and HCl as small aliquots of 2.5 M solutions. The experiments were started by the addition of 100 μl aliquots of [^{14}C]L-lysine (2.6 mM, 5 μCi/ml) (Radiochemical Centre, Amersham, Bucks.), to the temperature equilibrated systems (30°). Samples (2 × 1.0 ml) were removed at various time intervals for determination of uptake of radioactivity into whole cell and cold-trichloroacetic acid (TCA)-insoluble fractions; cold TCA-soluble uptake was obtained by difference. The samples removed for determination of total uptake were immediately filtered (Oxoid MF25, 0.45 μm pore size), washed with anaerobic suspension medium (2.0 ml), and removed to scintillation vials for radioactivity counting. The samples removed for determination of cold-TCA-insoluble uptake were immediately added to ice-cold 10% TCA (1.0 ml), kept on ice for 30 min, and the cell debris collected by membrane filtration. The filters were washed with ice-cold 0.25 mM TES, 0.1 mM Tris, pH 7.0 (4.0 ml) and removed for scintillation counting. 2.0 ml volumes of a scintillation fluid containing toluene, 500 ml; 1,4-dioxan, 250 ml; 2-methoxyethanol, 250 ml; naphthalene, 50 g; 2,5-diphenyloxazole, 4 g; *p*-bis-(2-(5-phenyloxazolyl)-benzene, 100 mg were used; the efficiency of counting was 73%.

2.3. Flame photometry

Intra- and extracellular potassium contents were obtained by removing 10 ml aliquots during directly comparable experiments. The samples were centrifuged as above, the supernatant fraction retained and the pellet resuspended to 10 ml in distilled water. These samples were assayed for potassium using a Gallenkamp flame photometer.

3. Results

Fig. 1a shows the uptake of [^{14}C]lysine into the cold-TCA-soluble fraction with cells buffered in TES-Tris at pH 7.2. The addition of 3,5,3′,4′-tetrachlorosalicylanilide (TCS) and valinomycin (VAL) (in this experiment during the assay, in others at zero time)

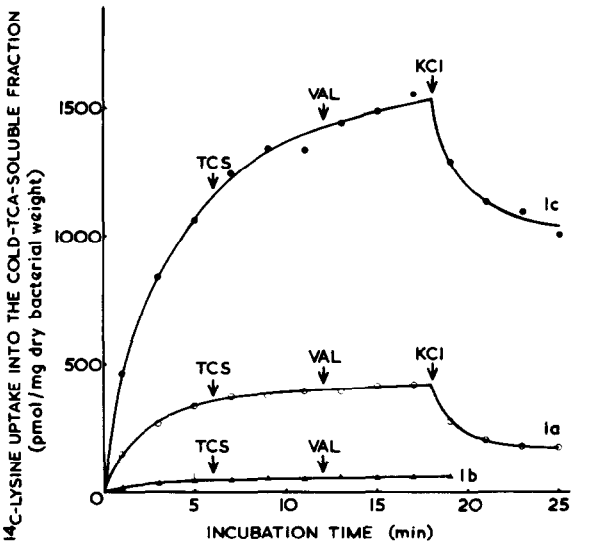

Fig. 1. Anaerobic *S. aureus* in (a) 10 mM TES, 4 mM Tris, pH ~ 7.0 (○—○—○); (b) 10 mM TES, 4 mM Tris, 400 mM KCl, pH ~ 7.0 (▲—▲—▲); (c) 0.25 mM TES, 0.1 mM Tris, pH ~ 7.0 (●—●—●). TCS was added to 1.6 × 10^{-6} M, valinomycin to 3.8 × 10^{-6} M and KCl to 100 mM and 50 mM in (a) and (c), respectively.

has no effect on either the time course or extent of uptake. If we assume that lysine is transported as a cationic species, the magnitude of the electrical potential across the membrane resulting in the disequilibrium of lysine, may be measured by substituting the values for the intra- and extracellular activities, or more approximately, concentrations of lysine, into the Nernst equation, $E = \frac{RT}{F} \ln \frac{[\text{Lys}]_i}{[\text{Lys}]_o}$. Taking the position at 18 min as an approximation to equilibrium, the distribution of counts gives uptake of lysine as 420 pmoles/mg dry bacterial weight and lysine equilibrium potential as 100 mV, the intracellular pool volume being taken as 1.55 ml/g dry bacterial weight [4] and the internal concentration of lysine at zero time as approx. 2 mM. When the relevant calculations were performed assuming various extents of exchange, it was found that when the appropriate correction factor was introduced for the specific activity of the [^{14}C]lysine, the calculated value for the lysine equilibrium potential remained effectively the same. In a parallel experiment, employing flame photometry, intra- and extracellular potassium concentrations were found to be

approx. 26.4 mM and 0.6 mM, respectively, giving an estimate of the potassium equilibrium potential as 98 mV. These figures also gave an estimate of the intracellular pH as 5.53.

On the addition of 100 mM potassium chloride during the assay, the potassium equilibrium potential, although not measured, would be greatly reduced or even reversed in polarity. Under these conditions a marked efflux of lysine is seen to occur, but a fraction (40%) of the intracellular lysine does not appear to be responsive to the change in potential and to remain within the cell. Although the washing medium did not contain the added potassium chloride we have demonstrated that the lysine efflux is not an osmotic effect.

In the presence of 400 mM potassium chloride (fig. 1b) where the potential would again be expected to be vanishingly small or reversed in polarity, uptake is seen to be severely restricted, presumably representing passive exchange at an initial rate of approx. 12 pmoles/mg dry bacterial weight/min.

These experiments were carried out in the presence of a relatively high Tris concentration (4 mM) which reduces the potassium equilibrium potential probably through the permeation of Tris base. Fig. 1c shows the uptake with cells in 0.1 mM Tris and a measured potassium equilibrium potential of 148 mV, the intra- and extracellular potassium concentrations being approx. 59.4 mM and 0.2 mM, respectively. The lysine equilibrium potential at 18 min is again in close agreement at 140 mV. Averaging these figures, and taking the measured external pH of 7.56, the intracellular pH is estimated as 5.16. On the addition of potassium chloride under these conditions, the fraction of lysine remaining within the cells increased to 67%.

Fig. 2 shows the effect of pH transitions on the rate of lysine uptake. At pH 5, the measured potassium equilibrium potential is only 29 mV and the rate of uptake 14 pmoles/mg dry bacterial weight/min. Addition of alkali to pH 7 raises the potential to 97 mV, and the rate is increased to 38 pmoles/mg dry bacterial weight/min. The rate is reduced on bringing the pH back to 5, and increased again by further addition of alkali.

At such relatively slow driving force, the rate limiting factor may be either the driving force or the actual membrane permeability for lysine. At pH 5 and 7, in the presence of 200 mM potassium chloride, exchange rates of approx. 13 and 17 pmoles/mg dry bacterial

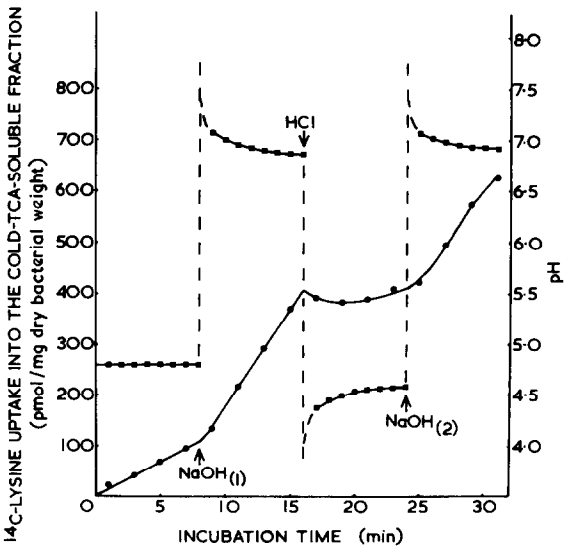

Fig. 2. Anaerobic *S. aureus* in 0.25 mM TES, 10 mM choline chloride, 0.42 mM HCl, pH ~ 5.0 containing 1.4×10^{-6} M TCS and 2.7×10^{-6} M valinomycin. (●—●—●) [^{14}C]lysine uptake; (■—■—■) pH. $NaOH_{(1)}$, HCl and $NaOH_{(2)}$ were added to 0.29 mM, 0.69 mM and 0.57 mM, respectively.

weight/min, respectively, were obtained. Under such conditions, therefore, where the membrane potential is negligible, the lysine permeability per se is effectively unaltered.

4. Discussion

It has been known for many years that the uptake of lysine into *S. aureus* is relatively insensitive to the action of proton translocating uncoupling agents, such as TCS [5, 6]. Such observations prompted Gale [6] to consider lysine uptake as a diffusion process, with the equilibrium resulting from a Donnan distribution. Following from this comment, and our predictions from the chemiosmotic hypothesis, two features are of particular significance; at neutral pH values lysine carries a net positive charge; it is experimentally possible to manipulate the membrane potential independently of metabolism.

When the cells used in these studies are allowed to go completely anaerobic as a result of nitrogen bubbling and their low endogenous respiratory activity, there is no evidence of further metabolism. Under such condi-

tions, there is no protonmotive force but the cells do possess a membrane potential. As a result of previous metabolic activity with its associated ion pumps, and the fixed negative charge within the cell, such resting cells retain high intracellular concentrations of cations, mainly potassium. These will tend to diffuse out of the cell down their concentration gradients, but in so doing will generate a potential across the membrane, inside negative. In response to this potential, other cationic species, for instance protons, will tend to move inwards. At electrochemical equilibrium, all diffusible ions will be distributed such that their gradient of chemical potential is balanced by the gradient of electrical potential. The presence of specific ionophores, such as TCS and valinomycin, will not alter the position of this equilibrium but simply ensure that the major permeant species responding to changes in the equilibrium are in fact protons and potassium. Under such conditions, the potassium equilibrium potential may be equated with the membrane potential. Thus, at Donnan equilibrium, the potassium equilibrium potential will be approximately equal to the proton equilibrium potential, or pH gradient. Also, in the presence of the appropriate membrane carrier, and assuming lysine to be transported as a monovalent cation, the potassium and lysine equilibrium potentials should have the same value. From the calculations based on the data in fig. 1, we see that this is indeed the case.

Prior to the addition of [^{14}C]lysine, the unlabelled lysine from the pool will be distributed across the cell membrane in a manner predicted by the prevailing membrane potential. The addition of the radioactive lysine results in an exchange process, and the distribution of the isotope as indicated above. The apparent lysine uptake observed in figs. 1a and 1c demonstrates such an equilibration process, the potentials with which the lysine is in equilibrium being 98 mV and 148 mV, respectively. The addition of TCS and valinomycin has no effect on this system, as these cells have already attained Donnan equilibrium due to their relatively high potassium and proton permeabilities.

The dependence of the isotope distribution on the potassium equilibrium potential becomes apparent when this potential is altered by the addition of a permeant ion to the suspending medium. The addition of potassium chloride after the uptake of [^{14}C]lysine (figs. 1a, 1c) would be expected to reduce the potassium equilibrium potential resulting in marked lysine efflux. The effect is, however, seen to be incomplete, 40% and 67% of the intracellular lysine respectively not responding to the depressed potential.

The presence of 400 mM potassium chloride prior to the [^{14}C]lysine (fig. 1b) would again be expected to reduce or even reverse the membrane potential; the added [^{14}C]lysine should therefore be distributed as predicted by this potential. What, in fact, is observed is an initial rapid rate followed by a much slower exchange process. This secondary slow exchange and the incomplete efflux observed in figs. 1a and 1c leads to the suggestion that this material represents a second intracellular pool of lysine bound, to some extent electrostatically, to material of fixed negative charge. A likely candidate for this material is the cellular DNA. This possibility in turn suggests a histone-like role for the otherwise high intracellular content of lysine.

The differences in the quantities of lysine bound in these experiments, however, needs further investigation. It seems unlikely that the different potassium concentrations employed to cause efflux would lead to such a discrepancy, as the logarithmic nature of the relationship between the potassium gradient and equilibrium potential, would render them almost equally effective. Variation in intracellular pH may also be discounted; in a parallel experiment in the presence of 0.1 mM Tris, and an external pH of 9.3, 67% of the lysine was found to be bound on the addition of potassium chloride to reduce the membrane potential. If at this extracellular pH, however, the Tris concentration was raised to approx. 13 mM, lysine efflux was found to be almost complete. It seems likely, therefore, that the bound lysine may be replaced by Tris to an extent dependent on the Tris concentration employed.

The membrane potential may also be modified by varying the external pH. For example, addition of alkali would result in proton efflux and potassium influx with consequent increase in the potential. The effect of such pH transitions on the rate of lysine uptake are shown in fig. 2. Increasing the potassium equilibrium potential by changing the pH from approx. 5 to 7 results in an increased rate of uptake; subsequent reduction in pH to 5 produces a decreased rate. The further addition of alkali gives the predicted increase in rate again.

The reduction of the potential (fig. 2) might have been expected to result in lysine efflux and not merely a decrease in uptake rate as observed. The effect is

probably a combination of a net efflux of lysine of low specific activity accompanied by the influx of lysine of higher specific activity by means of the exchange process.

The varying uptake rates in the two pH ranges may also be attributed to differences in the permeability coefficient for lysine transport. If, however, the membrane potential is clamped so that it is negligible, then any differences in the exchange rates of [^{14}C]lysine would be due to differences in the kinetics of permeation. We have demonstrated that such differences in the permeability coefficient in the pH ranges under investigation are minimal; we therefore conclude that the increased uptake rates observed in fig. 2 are largely the result of increasing the potassium equilibrium potential.

We are, however, aware that the results do not exclude the possibility that lysine base is the permeant species, responding to the pH gradient, rather than lysine cation, being dependent on the membrane potential. It would seem improbable, however, that lysine should permeate by this mechanism, as under normal metabolic conditions, when the intracellular pH is alkaline, with respect to that externally rather than acid as in these experiments, lysine exclusion would be manifest.

From these data therefore, we conclude that lysine is accumulated in *S. aureus* as a monovalent cation under the driving force of the membrane potential. Within the cell it equilibrates between the free amino acid pool and a fraction bound, possibly to DNA.

Acknowledgement

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