Feedback Inhibition of Pantothenate Kinase Regulates Pantothenol Uptake by the Malaria Parasite*

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To survive, the human malaria parasite Plasmodium falciparum must acquire pantothenate (vitamin B₅) from the external medium. Pantothenol (provitamin B₅) inhibits parasite growth by competing with pantothenate for pantothenate kinase, the first enzyme in the coenzyme A biosynthesis pathway. In this study we investigated pantothenol uptake by P. falciparum and in doing so gained insights into the regulation of the parasite’s coenzyme A biosynthesis pathway. Pantothenol was shown to enter P. falciparum-infected erythrocytes via two routes, the furosemide-inhibited “new permeation pathways” induced by the parasite in the infected erythrocyte membrane (the sole access route for pantothenate) and a second, furosemide-insensitive pathway. Having entered the erythrocyte, pantothenol is taken up by the intracellular parasite via a mechanism showing functional characteristics distinct from those of the parasite’s pantothenate uptake mechanism. On reaching the parasite cytosol, pantothenol is phosphorylated and thereby trapped by pantothenate kinase, shown here to be under feedback inhibition control by coenzyme A. Furosemide reduced this inherent feedback inhibition by competing with coenzyme A for binding to pantothenate kinase, thereby increasing pantothenol uptake.

The human malaria parasite Plasmodium falciparum cannot synthesize pantothenate and is completely dependent on its uptake from the external medium (2, 3). The membranes of uninfected human erythrocytes are largely impermeant to pantothenate (4), but the vitamin enters infected erythrocytes via the new permeation pathways (NPP) induced in the erythrocyte membrane after P. falciparum infection (4). Upon entering the host erythrocyte, pantothenate is taken up by the parasite via a low affinity H⁺-coupled transporter then, once in the parasite cytosol, is phosphorylated by the parasite’s pantothenate kinase (PfPanK) as the first step in its conversion to CoA (4, 5).

There have been several reports of antibacterial (6–8) and antimalarial (9, 10) pantothenate analogs. Recent recognition of the sequence dissimilarity between prokaryotic and eukaryotic CoA biosynthesis enzymes (11) has renewed interest in the CoA biosynthesis pathway as a target for the development of novel antibacterial agents (12–14). Similarly, recent evidence indicates that malaria parasite proteins required for pantothenate transport and metabolism hold promise as much-needed chemotherapeutic targets (15). The P. falciparum pantothenate transporter and PfPanK (neither of which have been characterized at a molecular level) differ from studied mammalian counterparts in their biochemical characteristics (e.g. substrate affinity) (5). The value of this pathway as an antimalarial target was validated by the discovery that the widely used provitamin pantothenol (which differs from pantothenate only in the replacement of the terminal carboxyl group with a hydroxyl group) as well as a range of other pantothenate analogs (16, 17) inhibit the growth of P. falciparum in vitro via a mechanism that involves competitive inhibition of pantothenate phosphorylation by PfPanK (3).

In this study we have investigated the mechanism of pantothenol uptake by P. falciparum-infected human erythrocytes and by parasites functionally isolated from their host cells by a saponin permeabilization technique. The results demonstrate that, like pantothenate, pantothenol gains access into infected erythrocytes via the NPP, although an additional pathway accounts for a minor component of its uptake. Once inside the erythrocyte, pantothenol is taken up across the parasite plasma membrane via a mechanism(s) that is functionally distinct from that which mediates the uptake of pantothenate. On entering the parasite, pantothenol is, like pantothenate, phosphorylated by PfPanK, which we show here to be inhibited by CoA. Furosemide was found to alleviate this CoA-mediated negative feedback inhibition of PfPanK and thereby increases the uptake of pantothenol by the parasite despite reducing its initial rate of

Pantothenate (vitamin B₅) is a precursor of coenzyme A (CoA), an obligate enzyme cofactor required in numerous metabolic processes. The five-step conversion of pantothenate to CoA is common to prokaryotes and eukaryotes, although the amino acid sequences of the enzymes involved vary considerably (1). The first step in this pathway is the conversion of pantothenate to 4’-phosphopantothenate by pantothenate kinase (PanK).² Plants, fungi, and various bacteria synthesize pantothenate de novo, whereas animals and certain microbes lack this ability and must acquire exogenous pantothenate (1).

* This work was supported by the Australian National Health and Medical Research Council Grant 224245 and by a grant from the UNICEF/UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases (TDR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: PanK, pantothenate kinase; PfPanK, P. falciparum PanK; BCCF, 2, 7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein; NPP, new permeation pathways; ESI, electrospray ionization; MS, mass spectrometry; pCMBS, p-chloromercuribenzenesulfonate; NEM, N-ethylmaleimide.

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uptake into \textit{P. falciparum}-infected erythrocytes by inhibiting the NPP.

**EXPERIMENTAL PROCEDURES**

Reagents—[\textsuperscript{14}C]Pantothenol (50 mCi/mmole) and [\textsuperscript{14}C]pantothenate (55 mCi/mmole) were purchased from American Radiolabeled Chemicals. [\textsuperscript{14}C]Choline (55 mCi/mmole) and [\textsuperscript{3}H]hypoxanthine (14.7 Ci/mmole) were purchased from American Biosciences.

Parasite Culture and Isolation—Experiments were performed using the 3D7 or FAF6 strains of \textit{P. falciparum}. The parasites were cultured in Group O, Rh\textsuperscript{-} erythrocytes and synchronized with sorbitol as described elsewhere (18). Parasites were “isolated” from their host erythrocytes by saponin treatment (4), which permeabilizes the cholesterol-containing erythrocyte plasma membrane and parasitophorous vacuole membrane (19) while leaving the parasite plasma membrane intact (20).

[\textsuperscript{14}C]Pantothenol and [\textsuperscript{14}C]Pantothenate Uptake across the Erythrocyte Membrane—The uptake of [\textsuperscript{14}C]pantothenol or [\textsuperscript{14}C]pantothenate into intact trophozoite-infected and uninfected erythrocytes was measured essentially as described previously for [\textsuperscript{14}C]pantothenate (4). Uptake was commenced by adding [\textsuperscript{14}C]pantothenol (2 \muM, 0.10 \muCi/ml in final reaction) or [\textsuperscript{14}C]pantothenate (2 \muM, 0.11 \muCi/ml) to the cell suspension and terminated by centrifuging (15,800 \times g, 2 min) 200-\muL aliquots of the cell suspension (~7 \times 10\textsuperscript{7} cells/ml for infected erythrocytes and between 7 \times 10\textsuperscript{7} and 3 \times 10\textsuperscript{9} cells/ml for uninfected erythrocytes) through 300 \muL of dibutyl phthlate, thus separating the cells from the supernatant solution containing the radiolabeled compound. Trophozoite-infected erythrocytes were separated from uninfected cells using the Miltenyi Biotec VarioMACS magnet (21, 22), giving a parasitemia of 95–97%. A cell volume of 75 fl was assumed for both infected and uninfected erythrocytes (4).

[\textsuperscript{14}C]Pantothenol, [\textsuperscript{14}C]Pantothenate, and [\textsuperscript{14}C]Choline Uptake into Isolated Parasites—The uptake of radiolabeled compounds by isolated trophozoites suspended in HEPES-buffered saline (125 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 20 mM glucose, 25 mM HEPES, pH 7.1 unless stated otherwise) at 37 °C was measured essentially as described previously (23). In experiments with ATP-depleted parasites, glucose was omitted from the saline (and the NaCl concentration was increased to 135 mM so as to maintain the osmolarity of the saline), and parasites were preincubated in this solution for 15–30 min at 37 °C. In the experiments giving rise to Fig. 5C, an inwardly negative membrane potential was imposed on ATP-depleted parasites as described previously (23). In all experiments the concentration of the extracellular radiolabeled compound was 2 \muM, and the cells were suspended at a density of ~10\textsuperscript{6} cells/ml. An isolated parasite volume of 28 fl was assumed (4). Furosemide, phloretin, N-ethylmaleimide (NEM), carbonyl cyanide \textit{m}-chlorophenylhydrazone, and valinomycin were added to the parasites (at the same time as the addition of radiolabel) as stock solutions in Me\textsubscript{2}SO, and an equal volume of Me\textsubscript{2}SO was added to the relevant controls (the maximum Me\textsubscript{2}SO concentration in the reaction solution was 0.4%, v/v). \textit{p}-Chloromercuribenzenesulfonate (pCMBS) was dissolved in water.

[\textsuperscript{14}C]Pantothenol and [\textsuperscript{14}C]Pantothenate Phosphorylation—The amount of [\textsuperscript{14}C]pantothenol and [\textsuperscript{14}C]pantothenate phosphorylated by PfPanK in parasite lysate (prepared from ~7 \times 10\textsuperscript{8} cells/ml) was measured under different conditions at pre-determined time points by precipitating phosphorylated compounds from solution using the Somogyi reagent (ZnSO\textsubscript{4} and Ba(OH)\textsubscript{2}), as described previously (4). The results of key experiments were confirmed with an alternate assay that relies on the separation of the phosphorylated from the unphosphorylated [\textsuperscript{14}C]substrate by binding the phosphorylated [\textsuperscript{14}C]substrate to DE81 filter disks followed by scintillation counting, as described previously (14, 24) with the following two modifications; the [\textsuperscript{14}C]substrate concentration was 0.1 Ci/ml (2 \muM), and the filter disks were washed 3 times, each time with 3 ml of an acetic acid:ethanol:water (1:95:4, v/v/v) mixture. In those experiments in which CoA and furosemide were added to the parasite lysate, they were added at the same time as the radiolabeled compound, and the relevant solvent (water and Me\textsubscript{2}SO) controls were included in each case.

Bio synthesis, Purification, and Analysis of 4'-Phospho-pantothenol—A mixture of pantothenol, ATP, MgCl\textsubscript{2}, and furosemide in 50 mM KH\textsubscript{2}PO\textsubscript{4} buffer, pH 7.4, was at time 0 combined with lysate prepared aseptically from ~3 \times 10\textsuperscript{8} \textit{P. falciparum} parasites, yielding a final volume of 1.8 ml and final concentrations as follows: pantothenol (1 mM), ATP (15 mM), MgCl\textsubscript{2} (15 mM), and furosemide (100 \muM). Furosemide was included in the reaction to alleviate feedback inhibition of PanK by CoA or CoA thiosterols that may have been present in the parasite lysate (see “Results”). The mixture was incubated for 48 h at 37 °C, and the reaction was terminated by transferring the mixture to 95 °C for 5 min. Precipitated protein was pelleted by centrifugation at 16,000 \times g for 5 min. A reaction mixture identical to the one described above but also containing 0.5 \muCi/ml [\textsuperscript{14}C]pantothenol was incubated at 37 °C for 48 h and processed in the same manner. The supernatant from this reaction was spotted on a plastic-backed 0.2-mm-thick silica gel 60 plate (Merck), and the plate was developed to 14 cm from the base line in a solvent of ethanol, 28% NH\textsubscript{4}OH (6:4, v/v). Horizontal strips of the thin-layer chromatography (TLC) plate (0.5 cm in width) were transferred to scintillation vials, to which 100 \muL of water followed by 3 ml of scintillation fluid was added. The vials were vortexed, and the radioactivity on each section of the plate was determined by scintillation counting. Scintillation counting revealed the production of a [\textsuperscript{14}C]-labeled pantothenol metabolite with an R\textsubscript{f} (0.61) distinct from that of pantothenol (R\textsubscript{f} = 0.88). The product of the unlabeled reaction could thereby be purified from unreacted pantothenol and other components in the supernatant by TLC. The reaction supernatant was applied in a band to a TLC plate, and the plate was developed to 14 cm from the base line in ethanol, 28% NH\textsubscript{4}OH (6:4, v/v). The region bracketing the product (R\textsubscript{f} = 0.61) was scraped from the plate, and the product was extracted from the silica gel by suspending the silica in water (5 times) and each time centrifuging at 14,000 \times g for 2 min. The water-soluble extracts were combined, concentrated in \textit{vacuo}, and subjected to \textsuperscript{1}H NMR and ESI-MS analysis. NMR spectra were recorded on a Bruker Avance 800 NMR spectrometer operating at 800.13 MHz. The chemical shifts (\delta) are reported as the shift in ppm.
**RESULTS**

**Pantothenol Is Taken Up by Infected and Uninfected Erythrocytes**—[14C]Pantothenol entry into human erythrocytes infected with *P. falciparum* trophozoites was initially rapid, reaching a distribution ratio ([pantothenol]i/[pantothenol]o) of 1 within 1 min, but slowing thereafter, reaching a distribution ratio of 2.2 ± 0.2 (mean ± S.E.) by 20 min (Fig. 1A, open circles). Furosemide (100 µM), an effective inhibitor of the NPP, reduced the initial rate of [14C]pantothenol uptake from a value ≥19 ± 3 µmol/(10¹² cells·h) to 2.7 ± 0.3 µmol/(10¹² cells·h) (p = 0.027), consistent with the NPP being the major but not the sole route for the uptake of pantothenol. However, furosemide significantly increased (approximately doubled; p = 0.036) the total accumulation seen over the 20-min time course (Fig. 1A, filled circles).

To determine whether the component of [14C]pantothenol uptake that was not inhibited by furosemide could be attributed to an endogenous erythrocyte pathway, we investigated [14C]pantothenol uptake by uninfected erythrocytes. Compared with [14C]pantothenate, which enters uninfected erythrocytes from trimethylsilyl-2,2,3,3-tetradeteropropionic acid (0.00 ppm). ESI-MS analysis was performed by the Research School of Chemistry Mass Spectrometry Facility, The Australian National University. Low resolution mass spectra were recorded on a Micromass-Waters LC-ZMD single quadrupole liquid chromatograph-mass spectrometer, and high resolution mass spectra were recorded on a Bruker Apex III 4.7T Fourier transform ion cyclotron resonance mass spectrometer using negative ion detection.

The product of the reaction was confirmed as 4′-phosphopantothenol by both 1H NMR and ESI-MS. 1H NMR (800 MHz, D₂O): δ 4.11 (s, 1H), 3.17 (dd, 1H), 3.65 (t, 2H), 3.47 (dd, 1H), 3.32 (t, 2H), 1.79 (m, 2H), 1.01 (s, 3H), 0.89 (s, 3H). MS (ESI) m/z: 284.2 ([M-H]⁻). High resolution mass spectrometry: calculated, 284.0899 for C₉H₁₉NO₇P; found, 284.0912.

**Measurement of Cytosolic pH**—The pH of the parasite cytosol was monitored using 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) as described elsewhere (20).

**Statistics**—Statistical comparisons were made using Student’s two-tailed t tests for paired or unpaired samples, as appropriate.
rocytes very slowly (Fig. 1B, open squares), \([^{14}C]\)pantothenol crossed the uninfected erythrocyte membrane rapidly (Fig. 1B, open circles), with an initial rate of \(4.2 \pm 0.4 \mu\text{mol/(10}^{12}\text{ cells/h)}\) or \([^{14}C]\)pantothenol accumulation after 20 min \((p = 0.9\) and 0.5, respectively; Fig. 1B, filled circles). Similarly, the addition of 10 mM pantothenol to the external medium had no significant effect on \([^{14}C]\)pantothenol uptake (not shown), consistent with pantothenol crossing the uninfected erythrocyte membrane either by simple diffusion through the membrane bilayer or through a low affinity transport system. An Arrhenius plot of the transport of pantothenol across the uninfected erythrocyte membrane (Fig. 1B, inset) was nonlinear and gave activation energies of 64 \(\pm 11\) kJ/mol at temperatures \(>25\) °C and 106 \(\pm 4\) kJ/mol at temperatures \(<25\) °C. Both the nonlinearity of the Arrhenius plot and the relatively high activation energies are consistent with a carrier-mediated process (25–28).

The pantothenol uptake rate observed in infected erythrocytes in the presence of furosemide is similar to the rate seen in uninfected erythrocytes, consistent with a fraction \((\approx 14\%\); i.e. the rate of pantothenol transport into infected erythrocytes in the presence of furosemide as a percentage of the transport rate in the absence of furosemide) of the pantothenol entering infected erythrocytes via an endogenous pathway, with the remainder \((\approx 86\%\) entering via the NPP.

\textbf{Pantothenol Is Taken up by Isolated Parasites and Phosphorylated—}The uptake of \([^{14}C]\)pantothenol by the intracellular parasite was investigated using parasites isolated by saponin permeabilization of the erythrocyte (and parasitophorous vacuole) membranes. The accumulation of \([^{14}C]\)pantothenol by isolated parasites reached a distribution ratio of 7.9 \(\pm 1.5\) after 20 min (Fig. 2), with an initial rate (estimated from the first time point) \(\approx 11 \pm 2\) mmol/(10^12 cells/h). The fact that the distribution ratio reached a value significantly \(>1\) might be explained either by an active transport process or by the metabolism of pantothenol within the parasite. Because pantothenol has been shown to act as a competitive inhibitor of the phosphorylation of pantothenate (3), we investigated whether pantothenol is itself phosphorylated by PfPanK using lysate prepared from isolated parasites. ^1H NMR and ESI-MS analyses revealed that incubation of pantothenol with parasite lysate resulted in the generation of \(^{4}\)-phosphopantothenol (see “Experimental Procedures” for details). We then compared the rate of pantothenol phosphorylation to that of pantothenate phosphorylation by parasite lysates. In paired experiments 62 \(\pm 2\%\) of \([^{14}C]\)pantothenol was phosphorylated after 40 min (Fig. 3, open circles) compared with 84 \(\pm 1\%\) of the same concentration of \([^{14}C]\)pantothenate (Fig. 3, open squares). The initial phosphorylation rates were 6.1 \(\pm 0.1\) mmol/
(10^{12} \text{ cells/h}) and 10 \pm 2 \mu M/(10^{12} \text{ cells/h}) for pantothenol and pantothenate, respectively. Similar results were obtained with an alternate assay for measuring pantothenate/pantothenol phosphorylation (Fig. 3, inset). Thus, pantothenol is phosphorylated by PfPanK but at a slower rate than pantothenate. Pantothenol uptake by the parasite, therefore, reflects a combination of transport and metabolism.

Pantothenol Uptake by Isolated Parasites Is pH-dependent—The transport of pantothenate across the parasite plasma membrane is pH-dependent (5). The pH dependence of [14C]pantothenol uptake by isolated parasites was investigated in parasites in which the contribution of the phosphorylation reaction to the measured uptake was inhibited by the inclusion of 500 \mu M unlabeled pantothenate in the solution. This concentration of pantothenate is sufficient to saturate PfPanK (which has a $K_m$ of 0.3 \mu M) (5) but should have little effect on the pantothenate transport mechanism (which has a $K_m$ of 23 mM) (5). Under these conditions both compounds equilibrated across the parasite plasma membrane, with pantothenol entering the parasite more rapidly than pantothenate ($p = 0.03$). The initial rates of pantothenate and pantothenol uptake were 4.2 \pm 0.1 \mu M/(10^{12} \text{ cells/h}) and \geq 16 \pm 2 \mu M/(10^{12} \text{ cells/h}) respectively (Fig. 4, open bars and filled bars, respectively). Furosemide did not significantly affect the transport rates of either compound ($p > 0.06$; Fig. 4B). Furthermore, ATP depletion did not affect the transport of pantothenol ($p = 0.07$; Fig. 4B) but caused a slight slowing of the initial rate of pantothenate transport to 2.5 \pm 0.1 \mu M/(10^{12} \text{ cells/h}) ($p = 0.01$; Fig. 4B).

Furosemide Increases Pantothenol Uptake by Isolated Parasites—The observation that furosemide increased pantothenol uptake by infected erythrocytes (Fig. 1A) but not by uninfected erythrocytes (Fig. 1B) is consistent with furosemide increasing pantothenol uptake by infected erythrocytes by acting on the sequestration of pantothenol within the parasite. This was tested directly. Furosemide greatly increased [14C]pantothenol uptake by isolated parasites (Fig. 4A), increasing the distribution ratio after 5 min from 3.6 \pm 0.3 to 13 \pm 2 ($p = 0.01$) and after 20 min from 7.9 \pm 1.5 to 25 \pm 4 ($p = 0.048$). By contrast, furosemide did not significantly increase the uptake of [14C]pantothenate after 5 min ($p = 0.9$; Fig. 4A, inset).

The effect of furosemide on the transport of pantothenol and pantothenate across the parasite plasma membrane was investigated in parasites in which the contribution of the phosphorylation reaction to the measured uptake was inhibited by the inclusion of 500 \mu M unlabeled pantothenate in the solution. This concentration of pantothenate is sufficient to saturate PfPanK (which has a $K_m$ of 0.3 \mu M) (5) but should have little effect on the pantothenate transport mechanism (which has a $K_m$ of 23 mM) (5). Under these conditions both compounds equilibrated across the parasite plasma membrane, with pantothenol entering the parasite more rapidly than pantothenate ($p = 0.03$). The initial rates of pantothenate and pantothenol uptake were 4.2 \pm 0.1 \mu M/(10^{12} \text{ cells/h}) and \geq 16 \pm 2 \mu M/(10^{12} \text{ cells/h}), respectively (Fig. 4B, open bars and filled bars, respectively). Furosemide did not significantly affect the transport rates of either compound ($p > 0.06$; Fig. 4B). Furthermore, ATP depletion did not affect the transport of pantothenol ($p = 0.07$; Fig. 4B) but caused a slight slowing of the initial rate of pantothenate transport to 2.5 \pm 0.1 \mu M/(10^{12} \text{ cells/h}) ($p = 0.01$; Fig. 4B).
extracellular pH (pH\textsubscript{o}) range 6.1 to 8.1. The corresponding intracellular pH (pH\textsubscript{i}) range is 7.07–7.72 (23). \[^{14}\text{C}]\text{Pantothenol} uptake showed a marked pH dependence, increasing with increasing pH\textsubscript{o} (Fig. 5A). There was a significant difference (p = 0.01) between the initial rates at pH 6.1 (0.7 ± 0.3 \text{\mu mol/(10}^{12} \text{cells-h}) and 8.1 (5.0 ± 1.0 \text{\mu mol/(10}^{12} \text{cells-h}). This is the opposite pH dependence from that seen with pantothenate (5), consistent with a fundamentally different uptake mechanism being involved.

The effect of pH on pantothenol uptake may reflect a pH dependence of the transport step or of the subsequent phosphorylation of the compound within the parasite. To determine whether pantothenol transport was associated with the movement of H\textsuperscript{+} equivalents, pH\textsubscript{i} was measured using the fluorescent pH indicator BCECF. The addition of 40 mM pantothenol to suspensions of BCECF-loaded isolated parasites did not affect pH\textsubscript{i} (Fig. 5B), consistent with pantothenol transport not being H\textsuperscript{+}-coupled. By contrast, the addition of 40 mM pantothenate caused a decrease in pH\textsubscript{i} (Fig. 5B), as reported previously (5).

It is also possible that changes in pH alter the conformation of pantothenol, affecting its ability to diffuse (or be transported) across the lipid bilayer. To investigate this possibility we used the Pallas for Windows software to predict the LogD (octanol/water distribution coefficient) over a wide pH range. The software predicted a LogD of 0.93 over the pH range of 0–11, only increasing when the pH was increased further. pH is, therefore, unlikely to alter the membrane permeability properties of pantothenol.

A decrease in pH\textsubscript{o} results in a depolarization of the parasite plasma membrane from its normal value of approximately −95 mV (18). An increase in the uptake of choline by the parasite at higher pH\textsubscript{o} values has been attributed to the dependence of choline influx on the membrane potential (23). Whether the membrane potential plays a role in pantothenol influx was tested in ATP-depleted parasites in which an inwardly negative membrane potential was imposed by the addition of the K\textsuperscript{+} ionophore valinomycin (1 \text{\mu M}) to parasites suspended in a low K\textsuperscript{+} medium. The imposition of an inwardly
FIGURE 6. Effects of pantothenate transport inhibitors on pantothenol uptake by isolated parasites. The effects of phloretin (200 μM), N-ethylmaleimide (NEM, 2 mM), p-chloromercuribenzenesulfonate (pCMBS, 10 μM), and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) on the uptake of 

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\text{[14C]pantothenol (2 μM; filled bars) and [14C]pantothenate (2 μM; open bars) by isolated parasites. The pantothenate transport inhibitors were added to the parasite suspension at the same time as the radiolabel, and the uptake was terminated after 5 min. The data are averaged from three-four separate experiments for pantothenol (shown + S.E.) and two-three separate experiments for pantothenate (shown + range/2 or S.E.). All experiments were performed in duplicate.}
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negative membrane potential increased the accumulation of 

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[14C]\text{choline but not [14C]pantothenol by ATP-depleted parasites (Fig. 5C).}
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Pantothenate Transport Inhibitors Do Not Inhibit Pantothenol Uptake—To confirm that the mechanisms of pantothenol and pantothenate uptake by the parasite are distinct, compounds shown previously to inhibit pantothenate transport (phloretin, carbonyl cyanide m-chlorophenylhydrazone, p-CMBS and NEM (5) were tested for their effect on pantothenol uptake by isolated parasites. None of these compounds had a significant effect on [14C]pantothenol uptake (p > 0.15; Fig. 6, filled bars), but all inhibited pantothenate uptake (Fig. 6, open bars), as shown previously (5). The data are consistent with pantothenol and pantothenate entering the parasite via separate mechanisms.

PfPanK Is Inhibited by CoA—Because furosemide and pH do not affect pantothenol transport by the parasite (Figs. 4B and 5B), we hypothesized that they affect pantothenol uptake by influencing its metabolism. This was tested by examining their effects on pantothenol phosphorylation in parasite lysate. Experiments using lysate provide a useful tool for studying phosphorylation independently of transport but do not take into account the regulation of phosphorylation in situ. CoA and/or its esters have been shown to inhibit the PanKs of bacteria, plants, and animals (1), although recently the PanKs from Staphyloccoccus aureus and Helicobacter pylori have been shown to be refractory to this feedback inhibition (29, 30). We therefore investigated the effects of CoA on PfPanK activity. 

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[14C]\text{Pantothenate phosphorylation by PfPanK was inhibited by CoA (Fig. 7) in a concentration-dependent manner. The IC}_{50}\text{ value (the concentration at which inhibition was half-maximal) was \sim 200 μM. Acetyl-CoA and malonyl-CoA were also effective inhibitors of pantothenate phosphorylation (data not shown).}
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Furosemide and pH Affect Feedback Inhibition of PfPanK—The effects of furosemide and pH on [14C]pantothenol phosphorylation by PfPanK in parasite lysate were tested both in the presence and absence of 400 μM CoA. In the absence of CoA, the addition of 100 μM furosemide had no significant effect on [14C]pantothenol phosphorylation (p = 0.1; Fig. 8A). However, the addition of 100 μM furosemide significantly alleviated the CoA-mediated inhibition of [14C]pantothenol phosphorylation by PfPanK (Fig. 8A), increasing phosphorylation 18.5-fold (p = 0.001). Similar results were obtained when the experiment was carried out with the alternate PanK assay (data not shown). In the absence of CoA, varying the pH in the range 7.07–7.72 (corresponding to the pH_{i} range 6.1–8.1) (23) had a small but significant effect on [14C]pantothenol phosphorylation (p = 0.022, comparing pH 7.07 and 7.72; Fig. 8B). The pH trend (i.e. decreasing phosphorylation rate with increasing pH in the absence of CoA) was opposite to that observed for uptake. However, CoA-mediated inhibition of [14C]pantothenol phosphorylation decreased with increasing pH (p = 0.028, comparing pH 7.07 and 7.72; Fig. 8C). Furosemide and pH had a similar effect on the phosphorylation of [14C]pantothenate in the presence of CoA (not shown).

Furosemide Is a Competitive Inhibitor of CoA-mediated PfPanK Inhibition—the mechanism by which furosemide alleviates the CoA-mediated inhibition of PanK was investigated further. We first tested the effect of increasing concentrations of furosemide on PanK activity in the absence of CoA. As can be seen in Fig. 9A, relatively high concentrations of furosemide (in the absence of CoA) inhibited PfPanK activity. We next carried out a kinetic analysis to determine the interaction between furosemide and CoA. Dixon plots revealed that furosemide antagonized the inhibitory effect of CoA on pantothenate (Fig. 9B) and pantothenol (Fig. 9C) phosphorylation by PfPanK.
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FIGURE 8. Effects of furosemide and pH on phosphorylation of pantothenol by PfPanK in parasite lysates. A, the effect of 100 μM furosemide on the phosphorylation of [14C]pantothenol (0.35 μM, measured over 20 min) in the presence and absence of 400 μM CoA. B and C, the effect of pH on the phosphorylation of [14C]pantothenol (0.35 μM, measured over 20 min) in the absence (B) and presence (C) of 400 μM CoA. The pH values shown (7.07, 7.30, 7.72) correspond to the predicted intracellular pH values upon exposure of parasites to the extracellular pH values 6.1, 7.1, and 8.1, respectively. The data are averaged from three separate experiments, each performed in duplicate, and are shown + S.E.

The growth and replication of the asexual form of the human malaria parasite, P. falciparum, is dependent on the uptake from the medium of pantothenate (2, 3), the universal precursor of the crucial cofactor CoA. Inhibitors of the conversion of pantothenate to CoA are being investigated both as antimalarial (3, 15–17) and antibacterial (12–14) agents. Pantothenol, a pantothenate analog and a common ingredient of multivitamin supplements, shampoos, and cosmetics, is one such candidate that has been shown recently to inhibit P. falciparum growth in vitro and to inhibit the in vivo proliferation of the mouse parasite Plasmodium vinckei vinckei. Pantothenol is a competitive inhibitor of the phosphorylation of pantothenate by PfPanK (3). Recently, additional pantothenate analogs have been synthesized and shown to inhibit P. falciparum proliferation at concentrations severalfold lower than those required to inhibit the proliferation of a human cell line (17). Like pantothenol, these compounds inhibit parasite proliferation via a mechanism that entails competition with pantothenate. Thus, compounds that increase the ratio of the concentration of the pantothenate analog (and perhaps its derivatives) to pantothenate in the intraerythrocytic malaria parasite may increase the potency of the analog. Combining the results from this study with the previous studies of pantothenate uptake (4, 5) has revealed two ways in which this might be achieved for pantothenol; that is, by restricting the access of pantothenol but not pantotheno into the intraerythrocytic parasite and by selectively increasing the uptake of pantothenol.

Fig. 10 shows a schematic representation of the processes involved in the uptake of pantothenol in P. falciparum-infected erythrocytes. Pantothenol gains access into the infected erythrocyte rapidly via the broad-specificity furosemide-sensitive NPP induced by the parasite in the host cell membrane (Fig. 10, step A) and also, at a slower rate, via an additional pathway (Fig. 10, step B). Because pantothenate uptake essentially relies on the NPP (4), whereas pantethenol can enter via an additional pathway, NPP inhibition may result in an increase in the intraerythrocytic [pantothenol]/[pantothenate] ratio. Having entered the erythrocyte, pantethenol is taken up by the intracellular parasite via a mechanism that differs in pH dependence and inhibitor sensitivity from the parasite pantothenate transport mechanism (Fig. 10, step C). Thus, the combination of pantothenol with a pantothenate transport inhibitor is likely to be synergistic. Unfortunately, the pantothenate transport inhibitors identified to date (5) lack the necessary specificity to allow this hypothesis to be tested directly. The observations that the transport mechanism of pantothenol is not coupled to H+ equivalents (Fig. 5B), is insensitive to sulfhydryl reagents (NEM and pCMBS, Fig. 6), and is not dependent on ATP (Fig. 4B), the membrane potential (Fig. 5C), Na+, or Cl− (not shown) are consistent with pantethenol crossing the parasite plasma membrane via a process of simple diffusion through the lipid bilayer. However, the activation energy for pantethenol transport across the uninfected erythrocyte membrane (Fig. 1B, inset) and its low LogD value are consistent with a carrier-mediated process (26, 31, 32), at least across this membrane. Further work needs to be carried out to determine the exact mechanism of pantethenol transport across the various membranes of the P. falciparum-infected erythrocyte.

On reaching the parasite cytosol, pantethenol is phosphorylated by PfPanK (Fig. 10, step D). It is not yet known whether phosphopantethenol continues to be metabolized by downstream enzymes to form CoA antimetabolites, as is the case for N-pentylpantothenamide in Escherichia coli (12). Furosemide and cytosol alkalinization partially alleviate the inherent feedback inhibition of PfPanK by CoA (Fig. 10, step E), thereby increasing pantethenol phosphorylation and consequently its uptake. Under similar conditions, furosemide does not have the same effect on pantethenol uptake (Fig. 4A, inset); furosemide might, therefore, be expected to increase the [pantothenol + derivatives]/[pantothenate] ratio inside the parasite.
FIGURE 9. Kinetic analysis of the effect of furosemide on PfPanK activity in the presence or absence of CoA. A, the effect of increasing the concentration of furosemide on [14C]pantothenol phosphorylation by PfPanK in parasite lysate in the absence of CoA. Dixon plots showing the effect of increasing furosemide concentrations on the CoA-mediated inhibition of pantothenate (B) and pantothenol (C) phosphorylation by parasite lysates. The data are averaged from three independent experiments, each performed in duplicate. The linearity of the phosphorylation reaction over the time course of the experiments was verified in each experiment (to control for variability in activity from different batches of lysate; data not shown). Error bars represent S.E. and, where not shown, fall within the symbols. For clarity, only positive error bars are shown in B and C. DMSO, Me2SO.
The observation that treatments that reduce CoA-mediated feedback inhibition of PfPanK affected pantothenol but not pantothenate uptake by isolated parasites (Fig. 4A) implies that the uptake of pantothenol is rate-limited by the rate of phosphorylation of the compound within the parasite, whereas the uptake of pantothenate is rate-limited by the transport across the parasite plasma membrane. The observations that the rate of pantothenate transport into isolated parasites is more than 3.5-fold higher than the rate of pantothenol transport into parasites is consistent with this hypothesis.

Several endogenous compounds have been shown previously to interfere with the feedback inhibition of PanKs. L-Carnitine was found to activate PanK from rat heart and reverses CoA-mediated feedback inhibition by an unknown mechanism (33). Very recently it was demonstrated that palmitoylcarnitine (and not L-carnitine) activated purified human PanK2 by competitively reversing the inhibition observed by acetyl-CoA (30). The authors suggested that both positive and negative regulation of PanK2 play a role in the functioning of the enzyme (30). The demonstration in our study that an unrelated exogenous compound (furosemide) is also able to competitively antagonize the inhibitory activity of the endogenous regulator (CoA) of PfPanK opens up the possibility that PanK regulation might be amenable to pharmacological intervention.

This study highlights the potential of targeting a pathway from two angles; (i) by impeding the access of a nutrient while allowing the access of analogs of the nutrient via an alternative route and (ii) by selectively increasing the uptake of an analog and its derivatives. Despite the very close structural similarity between pantothenate and pantothenol, their mechanisms of uptake are significantly different, providing opportunities for increasing the [pantothenol + derivatives]/[pantothenate] ratio in the intraerythrocytic malaria parasite with a second compound. Whether such a strategy is feasible in vivo remains to be seen.

Acknowledgments—We are grateful to the Canberra Branch of the Australian Red Cross Blood Service for the provision of blood and to John Allen, Gordon Lockhart, and Anitha Jayasingham of The Australian National University Mass Spectroscopy Facility for assistance with mass spectrometry.

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