Coenzyme A Requirement of Malaria Parasites: Enzymes of Coenzyme A Biosynthesis in Normal Duck Erythrocytes and Erythrocytes Infected with Plasmodium lophurae*

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ABSTRACT Normal duck erythrocytes and erythrocytes infected with Plasmodium lophurae have all of the enzymes for coenzyme A biosynthesis, whereas parasites freed from their host cells have none. Since erythrocyte-free cultivation of P. lophurae requires an exogenous source of coenzyme A, this parasite must obtain its coenzyme A entirely from the host cell during infection.

The avian malaria parasite Plasmodium lophurae, when maintained extracellularly in vitro, requires an exogenous source of coenzyme A (CoA) (1). This cannot be replaced by pantothenate or by the intermediates phosphopantothenate and phosphopantothenoylcysteine, but it can be partly replaced by phosphopantetheine and fully by dephospho-CoA (2). These findings suggested that the parasites lack at least part of the enzymes for the biosynthesis of CoA. In keeping with this idea, the demonstration that pantothenate kinase (ATP:pantothenate 4'-phosphotransferase, EC 2.7.1.-33), the first enzyme in this biosynthetic pathway, was present in both normal and P. lophurae-infected duck erythrocytes but absent from erythrocyte-free parasite preparations (3).

We now present evidence that all of the enzymes of CoA biosynthesis are present in host duck erythrocytes and that none can be detected in free parasite preparations. These results indicate that the CoA required by P. lophurae is supplied entirely by host cell metabolism and that these intracellular protozoan parasites lack the enzymes required for the conversion of pantothenic acid to coenzyme A.

MATERIALS AND METHODS

Materials. ATP, calcium pantothenate, and calf intestinal alkaline phosphatase were purchased from the Sigma Chemical Co., St. Louis, Mo. Diphospho-coenzyme A, coenzyme A, L-cysteine-hydrochloride, acetyl phosphate, dithiothreitol, and phosphorotransacetylase (Cl. kluveri) were all obtained from P-L Biochemicals, Inc., Milwaukee, Wisc. Micro Assay Culture Agar, Micro Inoculum Broth, and Pantothenate Assay Medium were purchased from Difeo Laboratories, Detroit, Mich. Cultures of Lactobacillus plantarum (ATCC 8014) and Lactobacillus helveticus (ATCC 12046) were obtained from the American Type Culture Collection, Rockville, Md. Both 4'-phosphopantothenyl-L-cysteine and 4'-phosphopantetheine were a generous gift from Dr. Yasushi Abiko of the Daiichi Suyaku Co., Tokyo, Japan.

4'-Phosphopantothenic acid was synthesized by the methods of King and Strong (4), as modified by Okada et al. (5). Paper chromatography of this compound in two different solvent systems gave a single spot with relative mobilities identical to published values (5). Quantitative assays for the phosphate and pantothenate moieties gave expected results.

Cell Isolation. Detailed procedures for maintaining heavy synchronous infections of these parasites and for isolating erythrocytes or host-free parasites have been described (6, 7). Giemsa-stained thin blood films were used to determine the extent of parasitemia (expressed as the total number of parasites per 100 erythrocytes) and the development stage of the parasite population. The erythrocytes and cell-free parasites used in these studies were obtained from control ducks, ducks infected with small parasites at the uninnucleate trophozoite stage of development (4th day after passage), and ducks infected with larger parasites at the multinucleate schizont stage of infection (5th day after passage). Only blood with heavy infections (90–130%) and with 80–90% of either uninnucleate or multinucleate parasites was used.

Enzyme Preparation. All operations were carried out at 4°. Washed, packed erythrocytes from control and infected ducks or host-free parasites were suspended in four times their packed cell volume of 0.05 M Tris-HCl buffer, pH 7.5, and passed through a French pressure cell at 8000 lbs./inch² (562.4 kg/cm²). Wet-mount slides of homogenized cells were observed with a microscope to estimate the degree of cell breakage. The homogenized cell suspensions were centrifuged at 13,000 X g for 0.5 hr in a Sorvall RC2-B refrigerated centrifuge, and the supernatants from this step were assayed directly for enzyme activity.

Assays. Phosphopantothenoylcysteine synthetase (pantothenate 4'-phosphate:L-cysteine ligase; EC 6.3.2.5), phosphopantothenoylcysteine decarboxylase [4'-phospho-N-(L-pantothenoyl)-L-cysteine carboxy-lyase; EC 4.1.1.36], dephospho-CoA pyrophosphorylase (ATP:pantetheine-4'-phosphate adenylyl-transferase; EC 2.7.7.3), and dephospho-CoA kinase (ATP:dephospho-CoA 3'-phosphotransferase; EC 2.7.1.24) were all measured by the procedures described by Abiko (8). Enzyme activities were proportional to the time of incubation and the amount of supernatant used in all of the assays. All enzyme assays were carried out at 41° in a temperature-controlled water bath. Pantothenic acid was determined by microbiological assays with L. plantarum (ATCC 8014), while

* This is paper no. 2 in the series. Paper no. 1 is ref. 2.
pantetheine was measured in a similar procedure with *L. helvetica* (ATCC 12046) (9). CoA was assayed by the phospho-
transacetylase method of Stadtman and Kornberg (10). Hemo-
globin was determined with the cyanomethemoglobin method.
Cell counts were done on a Hausser hemacytometer.

**RESULTS**

Throughout these investigations, only the supernatant frac-
tions of extracts from normal and infected red cells were ac-
tive for the enzymes measured. From comparable cell isolates,
extracts prepared by sonic disruption, freeze-thaw lysis, or
hypotonic lysis showed no variation in enzyme activity. No
change in activity was observed when phosphate buffer was
used in place of Tris buffer or when dithiothreitol was included
in the enzyme preparations. Dialysis of the supernatant frac-
tions did not alter enzyme activity.

When enzyme activities were expressed in a way that re-
fects the number of erythrocytes contributing to these ac-
tivities (Table 1), no significant differences were observed
between extracts prepared from normal erythrocytes and cells
infected with young, uninucleate schizonts. It was only
during the later stages of parasite development, when the
organisms were large, multinucleate schizonts, that differences
could be seen between extracts of normal and infected cells.
There was a decrease in the level of enzyme activity for every
enzyme measured. These differences were no longer apparent
when enzyme activities were expressed in terms of specific
activity (Table 2). Here, the specific activity of each enzyme
was essentially constant for all three sources of enzyme.

As was the case for normal and infected erythrocytes, a
variety of methods was used to prepare enzyme extracts from
host-free parasites. Whether these organisms were released
from their host erythrocytes by immune lysis or sapooin lysis,
no detectable activity for any of the CoA biosynthetic en-
zymes was observed, nor was activity observed in enzyme
assays using suspensions of intact organisms.

In view of these results, which held for both uninucleate and
multinucleate parasites, it was necessary to explain the activity
of phosphopantetheine and dephospho-CoA for the extra-
cellular development of *P. lophurae in vitro* (2). It seemed
likely that the duck erythrocyte extract, which is an essential
component of the culture medium, might convert phospho-
pantetheine and dephospho-CoA into sufficient amounts of
CoA to support parasite development. Accordingly, erythro-
cyte extracts prepared by freeze-thawing were incubated 4 hr
at 41° with (per ml) 2 μmol of ATP, 2 μmol of MgCl₂, 0.1
μmol of cysteine, and 0.05 μmol of pantothenate, phospho-
pantetheine, phosphopantetheine, phosphopantetheine, dephospho-
CoA, or CoA. The CoA levels found (Fig. 1) clearly show that both phosphopantetheine and dephospho-
CoA are rapidly converted to CoA within a period of 1.5 to 2
hr. Phosphopantetheine, on the other hand, never
generates more than 40% of the CoA concentration required
for optimum growth and development of host-free parasites
*in vitro*. With phosphopantetheine and pantetheine, the
former generates only 4% of optimum CoA concentrations,
while the latter yields no detectable CoA. Hence, phospho-
pantetheine and dephospho-CoA could replace CoA only
because they are rapidly converted to CoA by enzymes present
in the erythrocyte extract.

**DISCUSSION**

It has generally been found that when both malaria parasites
and their host erythrocytes contain the same enzymatic
activity, this activity is higher in infected than in uninfected
cells, whether expressed per cell or as specific activity. This is
the situation first shown by Sherman (11) for lactic dehydro-
genase of *P. lophurae* and since found for its pyruvate kinase

**Table 1. Enzyme activity per 10⁶ duck erythrocytes**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Phosphopantetheonyl-cysteine synthetase</th>
<th>Phosphopantetheonyl-cysteine decarboxylase</th>
<th>Dephospho-CoA pyrophosphorylase</th>
<th>Dephospho-CoA kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal duck erythrocytes</td>
<td>0.10 ± 0.01</td>
<td>2.34 ± 0.06</td>
<td>0.61 ± 0.05</td>
<td>2.37 ± 0.02</td>
</tr>
<tr>
<td>Infected erythrocytes (uninucleate parasites)</td>
<td>0.10 ± 0.02</td>
<td>2.23 ± 0.05</td>
<td>0.65 ± 0.03</td>
<td>2.44 ± 0.02</td>
</tr>
<tr>
<td>Infected erythrocytes (multinucleate parasites)</td>
<td>0.06 ± 0.01</td>
<td>1.28 ± 0.11</td>
<td>0.34 ± 0.04</td>
<td>1.28 ± 0.11</td>
</tr>
</tbody>
</table>

Activities are expressed as nmol/min per 10⁶ duck erythrocytes. The results are the average of three experiments and include the standard error.

**Table 2. Enzyme activity per mg of hemoglobin**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Phosphopantetheonyl-cysteine synthetase</th>
<th>Phosphopantetheonyl-cysteine decarboxylase</th>
<th>Dephospho-CoA pyrophosphorylase</th>
<th>Dephospho-CoA kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal duck erythrocytes</td>
<td>2.0 ± 0.3</td>
<td>48 ± 2</td>
<td>13 ± 1</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Infected duck erythrocytes (uninucleate parasites)</td>
<td>2.1 ± 0.4</td>
<td>45 ± 3</td>
<td>14 ± 1</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>Infected duck erythrocytes (multinucleate parasites)</td>
<td>1.8 ± 0.1</td>
<td>40 ± 5</td>
<td>12 ± 1</td>
<td>44 ± 5</td>
</tr>
</tbody>
</table>

The activities shown are nmol × 10⁶ per min/mg of hemoglobin. The results are the average of three experiments and include the standard error.
and phosphoglycerate kinase (12) and for its dihydrofolate reductase (13) and serine hydroxymethyltransferase (14).

Accordingly, it is highly significant that the specific activities (in relation to hemoglobin content) of all the enzymes of CoA biosynthesis were the same in infected as in uninfected duck erythrocytes. When large parasites were present (in 5-day infections) that had ingested and digested appreciable amounts of host cell cytoplasm (15), the enzyme activities per erythrocyte were decreased but the specific activities remained constant. A similar pattern has already been shown for the folate enzymes 10-formyl tetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase, enzymes which, like those of CoA biosynthesis, could not be found in parasites freed from host cells (14).

It must, of course, be recognized that failure to demonstrate an enzymatic activity may result from its inactivation during preparation rather than from its absence in the cell under study. In view, however, of our inability to find in free *P. lophurae* any of the five enzymes of CoA biosynthesis by any of three methods of preparation, in view of the ready demonstration of all of these enzymes in extracts of infected as well as uninfected host erythrocytes, and in view of the CoA requirement of parasites developing extracellularly in vitro (2), we feel confident in concluding that erythrocyte stages of *P. lophurae* lack the enzymes for CoA biosynthesis.

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