Several lines of evidence demonstrating that *Plasmodium falciparum*, a parasitic organism, has distinct enzymes for the phosphorylation of choline and ethanolamine

Marie L. Ancelin and Henri J. Vial

CNRS UA.530-INSERM U.58, 60 Rue de Navacelles, 34100 Montpellier, France

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In *Plasmodium falciparum*-infected erythrocyte homogenates, the specific activity of ethanolamine kinase (7.6 ± 1.4 nmol phosphethanolamine/10^7 infected cells per h) was higher than choline kinase specific activity (1.9 ± 0.2 nmol phosphocholine/10^7 infected cells per h). The K_m of choline kinase for choline was 79 ± 20 μM, and ethanolamine was a weak competitive inhibitor of the reaction (K_i = 92 mM). Ethanolamine kinase had a K_m for ethanolamine of 188 ± 19 μM, and choline was a competitive inhibitor of ethanolamine kinase with a very high K_i of 268 mM. Hemicholinium 3 inhibited choline kinase activity, but had no effect on ethanolamine kinase activity. In contrast, D-2-amino-1-butanol selectively inhibited ethanolamine kinase activity. Furthermore, when the two enzymes were subjected to heat inactivation, 85% of the choline kinase activity was destroyed after 5 min at 50°C, whereas ethanolamine kinase activity was not altered. Our results indicate that the phosphorylation of choline and ethanolamine was catalyzed by two distinct enzymes. The presence of a de novo phosphatidylethanolamine Kennedy pathway in *P. falciparum* contributes to the bewildering variety of phospholipid biosynthetic pathways in this parasitic organism.

Malaria (Plasmodium falciparum) *Choline kinase* *Ethanolamine kinase* *Phospholipid*

1. INTRODUCTION

Plasmodium is a protozoon, which is the agent of malaria, one of the most lethal parasitic diseases in the world. During the pathological erythrocytic stage, there is a considerable increase (as much as 500%) in the erythrocyte phospholipid content, especially PC and PE [1,2]. We have previously detected and quantified the different pathways of phospholipid metabolism in *P. knowlesi* [3], as well as in *P. falciparum*-infected erythrocytes [4].

Parasitized erythrocytes are capable of incorporating high amounts of labeled ethanolamine. However, the rate of PE formation by PS decarboxylation is enough to satisfy most of the parasite requirements for this phospholipid. Since ethanolamine is present in plasma at very low levels [5] and quasi-absent from the culture medium of *P. falciparum* [6], labeled ethanolamine incorporation could merely reflect either a Ca^{2+}-dependent base-exchange mechanism or a utilization of ethanolamine instead of choline, via the PC Kennedy pathway [7].

The problem is crucial since PE is the phospholipid most increased in absolute amounts, after infection by *Plasmodium*. However, until now, it has never been demonstrated that a biosynthesis of PE via the Kennedy pathway also exists in situ in *Plasmodium*-infected erythrocytes.

Here, we investigate the characteristics of ATP:choline phosphotransferase (choline kinase,

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PhoCh, phosphorylcholine; PhoEth, phosphorylethanolamine

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EC 2.7.1.32) and ATP:ethanolamine phosphotransferase (ethanolamine kinase, EC 2.7.1.82), the first-step catalyzing enzymes of the de novo synthesis of PC and PE, respectively. The nearly complete absence of competition between the two types of substrate for the enzymes, as well as the characteristics of these two enzymatic activities under various conditions, clearly indicate that two distinct enzymes exist and that there is a biosynthesis of PE by the Kennedy pathway in situ in the parasitic protozoon *P. falciparum*.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[*methyl*-^{14}C]Choline and [2-^{14}C]ethan-1-ol-2-amine were purchased from Amersham. Choline chloride, ethanolamine, hemicholinium 3, were supplied by Sigma, and D-2-amino-1-butanol came from Ventron. Dowex AG 1X8 (chloride form), 100–200 mesh, was obtained from Biorad and RPMI 1640 from Gibco (France). All reagents were of analytical grade.

2.2. *Biological material*

AB^+ human blood or AB^+ human serum came from the Blood Bank of Montpellier (France).

The Nigerian strain of *P. falciparum* (Dr W.H. Richard, Wellcome Research Laboratories, Beckenham, England [8]) was maintained by serial passages in AB^+ human erythrocytes suspended in complete medium (RPMI 1640 supplemented with 25 mM Hepes buffer, pH 7.4, and 10% AB^+ serum) at 37°C, using the petri-dish candle-jar method [6]. For experimentation, infected cells were pelleted at 7000 × g·min, then lyzed by an equal volume of water and further sonicated in a probe-type sonicator (Branson B 30 sonifier) for 30 s at 4°C. Homogenates could be stored at −20°C for several days. In some experiments, the crude hemolysate of infected erythrocytes was further fractionated to separate the parasite cytosol fraction from the parasite membrane structures, as described in [9].

2.3. *Enzyme assay*

Choline kinase (ATP:choline phosphotransferase) (EC 2.7.1.32) was determined as in a previous characterization done in infected erythrocytes [9]. Unless otherwise specified, the incubation mixtures contained 125 mM Tris-HCl, pH 7.9, 10 mM ATP, 10 mM $MgCl_2$, 5 mM EGTA, 0.5 mM [*methyl*-^{14}C]choline (0.9 Ci/mol) and 20 μl of enzyme extract in a final volume of 200 μl. After 45 min at 37°C, the reaction was stopped at 4°C. Routinely, each reaction mixture was applied to a column (0.55 × 2.5 cm) of Dowex AG 1X8, OH^- form, 100–200 mesh, ion-exchange resin. After washing with 10 ml water, PhoCh was eluted with 0.5 ml of 1 N NaOH, followed by 1.5 ml of 0.1 N NaOH. In preliminary experiments, cellular lipids were extracted as in [10] to verify that neither CDP-choline nor PC was formed during the incubation procedure.

Ethanolamine kinase (ATP:ethanolamine phosphotransferase, EC 2.7.1.82) was determined according to Weinhold and Rethy [11]. Incubation mixtures contained 60 mM Tris-HCl, pH 8.5, 3 mM ATP, 3 mM $Mg(CH_3COO)_2$, 0.2 M KCl, 1.2 mM [2-^{14}C]ethanolamine (2.2 Ci/mol) and 20 μl of enzyme in a final volume of 200 μl. The reaction was started by adding the enzyme extract, and after 45 min at 37°C, it was stopped by cooling to 4°C and adding 50 μl ethanol. PhoEth was separated from ethanolamine by paper chromatography (Whatman 3MM) in 2.7 M ammonium acetate buffer, pH 5.0/95% ethanol (3–7, v/v) [12]. Authentic standards were also applied for migration. After visualization by a ninhydrin spray, the appropriate spots were cut from the paper chromatograms and counted after addition of scintillation cocktail (Packard no.299) in a Packard 460 CD spectrometer.

Results are expressed as nmol/10^7 infected cells per h, since results expressed as nmol/mg protein would lead to imprecise and irreproducible results depending on parasitemia (i.e. the percentage of infected cells) of the original suspension.

3. RESULTS

In previous studies, we have determined the conditions of choline kinase assay in *P. falciparum*-infected erythrocytes [9]. Ethanolamine kinase activity was here assayed according to Weinhold and Rethy [11]. Under our assay conditions, radioactive choline and ethanolamine were only recovered, after incubation, as PhoCh and PhoEth, respectively. We particularly checked that

neither CDP-choline (or CDP-ethanolamine), nor PC (or PE) was formed.

P. falciparum-infected erythrocyte homogenates phosphorylate ethanolamine more actively than choline, at specific activities of 7.6 ± 1.4 (n = 10), and 1.9 ± 0.2 (n = 15) nmol/10^7 infected cells per h, respectively. Very little kinase activity was found in normal uninfected erythrocytes: 70 pmol PhoEth/10^7 cells per h and 6 pmol PhoCh/10^7 cells per h, which is less than 1% of the activity of infected cells. Therefore, results are expressed as nmol product per infected cells.

After fractionation of *P. falciparum*-infected erythrocyte homogenates, choline and ethanolamine kinase activities were specifically located in the cytosol fraction and neither activity was recovered in the membrane fraction. A quite comparable activity was found in the crude homogenate (not shown). Therefore, we routinely assayed choline and ethanolamine kinase activities in infected erythrocyte homogenates.

When ethanolamine kinase was assayed under the same conditions as choline kinase (i.e. at 10 mM ATP and $MgCl_2$, pH 7.9) the activity of ethanolamine kinase was not significantly different, whereas when choline kinase was assayed under the same conditions as ethanolamine kinase (3 mM ATP and $Mg(CH_3COO)_2$, pH 8.5), the activity of choline kinase was 57% reduced, indicating that the conditions for ethanolamine kinase assay could not be applied to the assay of choline kinase.

When the homogenates were incubated with various concentrations of β-mercaptoethanol (from 0.1 to 40 mM), no increase in choline kinase activity was found, differing from choline kinase activity in yeast [13]. With the same β-mercaptoethanol concentrations, ethanolamine kinase activity was also unchanged.

3.1. *Influence of substrate concentrations: cross-inhibition studies*

The apparent K_m of choline kinase for choline was 79 ± 20 μM (n = 6), whereas the K_m of ethanolamine kinase for ethanolamine was slightly higher, 188 ± 19 μM (n = 3). A possible competition between the two types of substrate for the enzymes was investigated. Fig.1A shows that

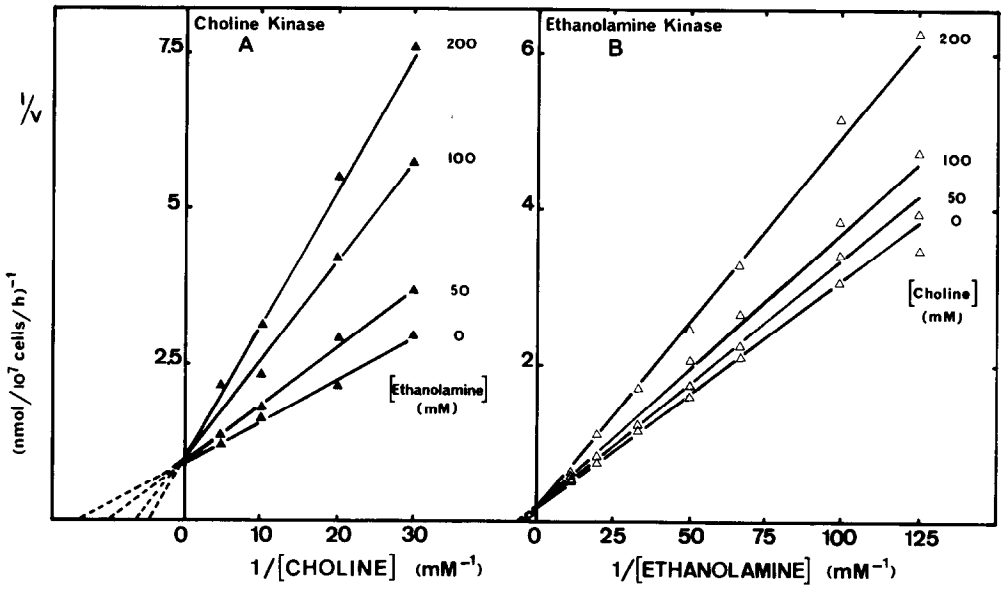


Fig.1. Substrate competition studies for choline and ethanolamine kinase activities in *P. falciparum*-infected erythrocytes. Double-reciprocal plots of the initial velocities of: (A) choline kinase in the presence of increasing ethanolamine concentrations (▲—▲); the homogenate fraction contained 1.7 × 10^7 infected cells per assay; (B) ethanolamine kinase in the presence of increasing choline concentrations (△---△); the homogenate fraction contained 1.35 × 10^7 infected cells per assay.

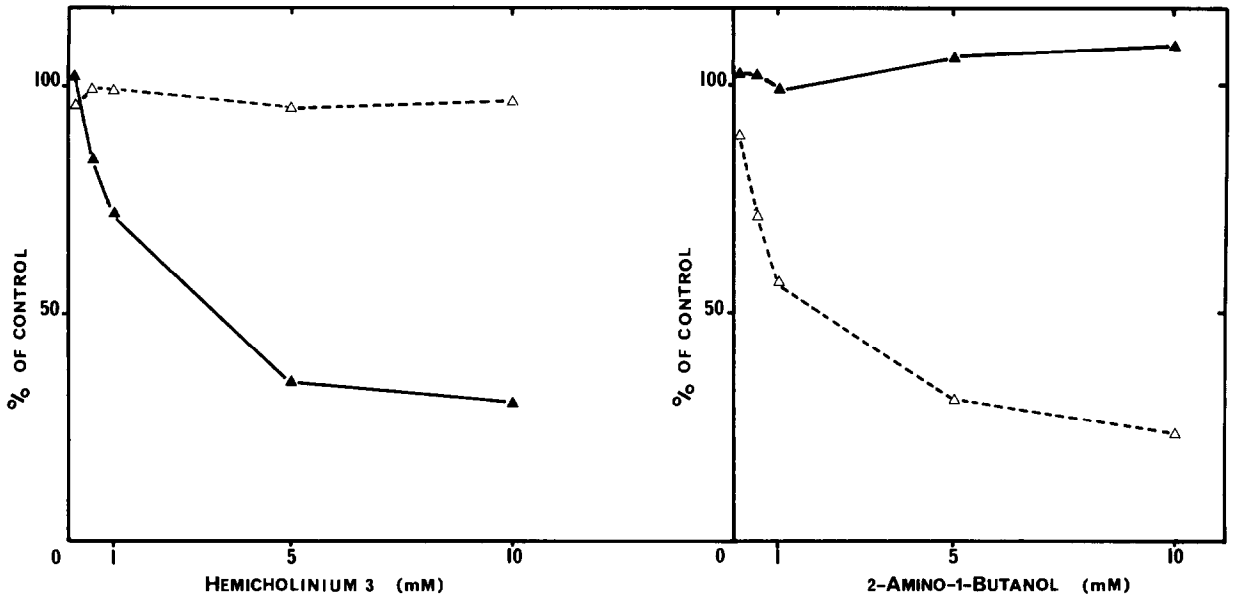
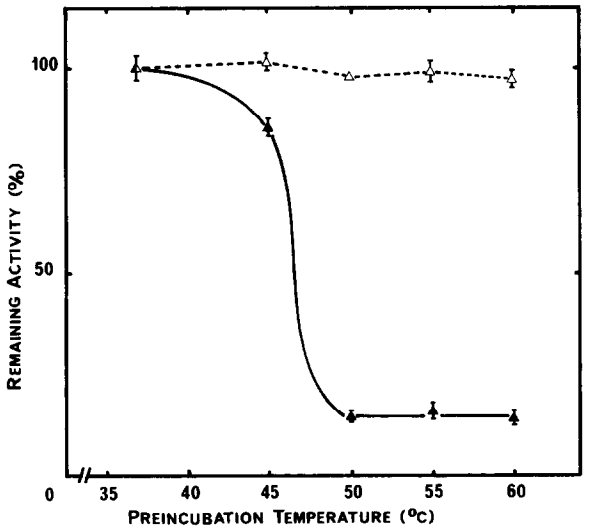


Fig.2. Differential inhibition of choline and ethanolamine kinases by hemicholinium 3 and D-2-amino-1-butanol. The extracts, containing 10^7 infected cells, were incubated as described in section 2, except that the indicated concentrations of hemicholinium 3 or D-2-amino-1-butanol were added. Results are the means of two experiments each carried out in duplicate. Control activities were 2.6 ± 0.1 and 18.0 ± 0.3 nmol/10^7 infected cells per h for choline kinase (▲—▲) and ethanolamine kinase (△---△), respectively.

ethanolamine was a competitive inhibitor of choline kinase. However, the K_i of 92 mM, obtained from linear extrapolations of a replot of the Lineweaver-Burk slopes vs ethanolamine concentration [14], indicates a very low affinity of this substrate for choline kinase. Similarly, choline was a very weak competitive inhibitor of ethanolamine kinase, with a high K_i of 268 mM (fig.1B). Therefore, in both cases, choline and ethanolamine kinases have very high specificities for their natural substrate, without any appreciable affinity for ethanolamine or choline, respectively.



3.2. *Differential inhibition of choline and ethanolamine kinases by two structural analogs*

Fig.2 shows that hemicholinium 3 significantly and specifically decreased choline kinase activity

Fig.3. Heat-inactivation of choline and ethanolamine kinases of *P. falciparum*-infected erythrocytes. Homogenates of infected erythrocytes (7.7×10^6 parasitized cells per assay) were preincubated for 5 min at the specified temperatures, then removed and assayed at 37°C for kinase activities as described in section 2. Duplicate experiments were repeated at least twice. Control activities were 1.19 ± 0.02 and 3.6 ± 0.2 nmol/10^7 infected cells per h for choline kinase (▲—▲) and ethanolamine kinase (△---△), respectively.

by 16% at 0.5 mM to as much as 70% at 10 mM. But similar concentrations of hemicholinium 3 had no effect on ethanolamine kinase activity. In contrast, D-2-amino-1-butanol did not affect choline kinase activity whereas ethanolamine kinase was reduced by 10 to 76% in the range of 0.1 and 10 mM, respectively.

3.3. *Heat inactivation*

When homogenates were preincubated at 45°C for various periods and assayed for kinase activities at 37°C, both of the kinase activities were destroyed at the same rate, with 50% of the activity remaining after 30 min of preincubation. However, in other experiments, the homogenates were preincubated for 5 min at various temperatures, ranging from 37 to 60°C, before assaying for kinase activities at 37°C. Fig.3 shows that choline kinase activity was 85% destroyed after 5 min at 50°C, whereas ethanolamine kinase was very resistant to heat, since 90% of the activity was still recovered after preincubation at 60°C.

4. DISCUSSION

The major finding of this study is the clear evidence that choline kinase and ethanolamine kinase activities are separate in the protozoon *Plasmodium*. The apparent K_m values of choline and ethanolamine kinases for choline and ethanolamine do not differ greatly: 79 ± 20 μM (n = 6) and 188 ± 19 μM (n = 3), respectively. However, experiments designed to test for competition between the two substrates for each enzyme activity clearly indicate that choline and ethanolamine phosphorylations involve two distinct active sites. The K_i of choline kinase inhibition by ethanolamine was more than 1100-times as high as the K_m of choline kinase activity, already indicating that the catalytic site of choline kinase has a quite low affinity for ethanolamine. Similarly, the catalytic site of ethanolamine kinase is also unable to recognize choline effectively, since the K_i of ethanolamine kinase inhibition by choline was more than 1400-times as high as the K_m of ethanolamine kinase for ethanolamine.

Wittenberg and Kornberg [13] have shown that decreasing the number of *N*-methyl groups in choline increases the K_m of choline kinase for these substrates. Hence, the 3 methyl groups appear to be crucial for the transformation of choline by choline kinase. Similarly, our experiments show that the primary amine group of ethanolamine seems to be essential to its phosphorylation by ethanolamine kinase. In fact, it should be noted that, under our conditions of pH assay, choline had a permanent charge, and that 84% of the ethanolamine was in the protonated form at pH 8.5 (pK = 9.2 at 36°C [15]). This indicates that the high specificity of the two kinases for their respective substrates cannot result from a mere charge phenomenon.

Several studies have previously reported the ability of choline kinase preparations to phosphorylate ethanolamine at much lower rates [12,16,17]. Our data show that under our defined and differing assay conditions, extracts of *P. falciparum*-infected erythrocytes phosphorylated ethanolamine at a higher rate (more than 4-fold) than choline. Furthermore, when assayed under the same conditions as ethanolamine kinase, choline kinase activity was decreased by 57%. Thus, the two kinase activity require different assay conditions, already suggesting the involvement of two enzyme catalytic sites.

Other evidence for two distinct kinase activities was provided by their differential inhibition by two structural analogs. Hemicholinium 3, which like choline has a quaternary ammonium group, interferes with various reactions involving choline as substrate, such as acetylation in the nervous system [18], phosphorylation in various tissues [19,20], and transport of choline in erythrocytes [21,22]. Hemicholinium 3 specifically inhibited choline kinase activity but had no effect on ethanolamine kinase activity. In contrast, D-2-amino-1-butanol, which we previously reported as an inhibitor of PE biosynthesis in *Plasmodium*-infected erythrocytes [23], selectively inhibited ethanolamine kinase activity.

Lastly, choline and ethanolamine kinase showed quite different stabilities after heating. Most of the choline kinase was destroyed by heating, whereas ethanolamine kinase activity was entirely recovered after 5 min at 60°C (see fig.3). Hence, this difference in stability clearly distinguishes the two kinase activities.

Although the two kinase activities were not physically separated, the many lines of evidence

provided by our study clearly demonstrate that two distinct enzymes are involved in the phosphorylation of choline and ethanolamine in *Plasmodium*-infected erythrocytes.

Until now, a de novo biosynthesis of PE via the Kennedy pathway has never been demonstrated in this parasite. In a previous work, using a wide variety of approaches, we were unable to distinguish between the two last-step catalyzing enzymes in the de novo synthesis of PC and PE. Nevertheless, it appears that *Plasmodium* contains the necessary enzymatic machinery for synthesizing PC and PE [24]. The high level of a specific ethanolamine kinase activity shown here, as well as the high incorporation of labeled ethanolamine into PE by *Plasmodium*-infected erythrocytes [3], strongly suggest that a de novo PE biosynthesis by the Kennedy pathway takes place in situ in parasitized cell.

A question remains as to the role of the PE Kennedy pathway in this hematozoon, whose PS decarboxylation activity would appear to be sufficient to satisfy for the whole PE requirement. Do the two pools of PE biosynthesized either from incorporated ethanolamine or by PS decarboxylation, and the two pools of PC biosynthesized either from incorporated choline or by methylation of PE [3], correspond to specific locations or particular functions in the parasite?

The protozoon *Plasmodium* appears to be midway between the prokaryotes, whose PE synthesis results exclusively from the decarboxylation of PS [25–28], and multicellular organisms (higher eukaryotes) which possess specific ethanolamine kinase activity [29,30] and synthesize PE only by the de novo Kennedy pathway [31–33]. Until now, to our knowledge, the existence of a de novo biosynthesis of PE in a parasite has not been demonstrated, but this constitutes a crucial element in the study of phospholipid metabolism in parasitic organisms. The single-cell eukaryote, *Plasmodium*, exhibits a bewildering versatility in the synthesis of its phospholipid and appears to be as intricate as many multicellular organisms.

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