Chloroquine Binds in the Cofactor Binding Site of Plasmodium falciparum Lactate Dehydrogenase*

(Received for publication, August 25, 1998, and in revised form, December 8, 1998)

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Although the molecular mechanism by which chloroquine exerts its effects on the malarial parasite Plasmodium falciparum remains unclear, the drug has previously been found to interact specifically with the glycolytic enzyme lactate dehydrogenase from the parasite. In this study we have determined the crystal structure of the complex between chloroquine and P. falciparum lactate dehydrogenase. The bound chloroquine is clearly seen within the NADH binding pocket of the enzyme, occupying a position similar to that of the adenyl ring of the cofactor. Chloroquine hence competes with NADH for binding to the enzyme, acting as a competitive inhibitor for this critical glycolytic enzyme. Specific interactions between the drug and amino acids unique to the malarial form of the enzyme suggest this binding is selective. Inhibition studies confirm that chloroquine acts as a weak inhibitor of lactate dehydrogenase, with mild selectivity for the parasite enzyme. As chloroquine has been shown to accumulate to millimolar concentrations within the food vacuole in the gut of the parasite, even low levels of inhibition may contribute to the biological efficacy of the drug. The structure of this enzyme-inhibitor complex provides a template from which the quinoline moiety might be modified to develop more efficient inhibitors of the enzyme.

Malaria is one of the major diseases of mankind, claiming 3 million lives worldwide annually. Resistance to existing antimalarial drugs is a large and increasing problem. Recently we have determined the first high-resolution structure of an enzyme from the Plasmodium falciparum parasite, the causative agent of malaria. This 1.7-Å structure (1) of the essential glycolytic enzyme P. falciparum lactate dehydrogenase (pfLDH) has revealed a unique cleft adjacent to the active site, ideally suited as a target for the rational design of inhibitors. Another feature of the structure is a significant displacement of the NADH cofactor relative to other forms of LDH, reflecting that the malarial enzyme has a unique mode of association with the cofactor and hence a distinctive NADH binding pocket. These features suggest pfLDH may form an appropriate target for structure-based design of novel antimalarials.

Chloroquine (CQ, Fig. 1, left) and related quinoline compounds have been used extensively throughout the world as prophylactics to prevent the development of malaria. Although the mechanism of action of CQ on the parasite is not completely understood, it is thought to interfere with the function of the food vacuole in the mature stages of the erythrocytic parasite (2). CQ is a weak base and accumulates to high concentrations within the acidic food vacuole (3). Within these vacuoles, hemoglobin is degraded by proteases to provide a supply of amino acids for the parasite, but also producing toxic heme moieties as a by-product (hematin; Fig. 1, right). The parasite normally detoxifies hematin by polymerizing the by-product to hemozoin, although the details of this process are unclear. Because CQ is known to bind to hematin, it was thought to exert its antimalarial activity by forming a complex with hematin, which inhibits its sequestration into hemozoin (4). Free heme causes cell lysis, and so CQ complexation with hematin was thought to be toxic to the parasite. This hypothesis is no longer favored because of the failure to show the existence of free heme in parasite cells. It has also been suggested that the target for CQ is a protein or proteins involved in the digestion or disposal of hemoglobin (5), although no target has been identified. CQ resistance is an increasingly serious problem and appears to correlate with decreased accumulation of the drug by CQ-resistant parasites. Identifying and understanding the interaction of proteins with CQ could lead the way to the development of new antimalarials to overcome these problems.

Intriguingly, recent studies (6) showed that photoreactive analogues of chloroquine interacted specifically with two proteins in infected red blood cells, one of which was identified (7) as pfLDH. In these studies some evidence for CQ binding to pfLDH was found, but it was not observed to inhibit the activity of pfLDH. However, CQ was found to modulate the inhibitory effect of hematin on LDH. Hematin itself strongly inhibits the malarial form of the enzyme ($K_i = 0.2 \mu M$), about 100-fold tighter than its binding to mammalian forms of the enzyme ($K_i = 25 \mu M$ for bovine LDH). Equimolar amounts of CQ modestly decreased inhibition of the mammalian form by hematin by about 50% ($K_i = 33 \mu M$), whereas inhibition of the malarial enzyme was reduced by at least 2 orders of magnitude in the presence of CQ. This protective effect of CQ is most likely because of the formation of a tight complex between CQ and hematin (8), this complex binding less strongly to LDH than does free hemin itself.

In this study the crystal structure of the complex formed between chloroquine and pfLDH has been determined to assess the mode and the likely significance of this interaction. Additionally, the structure of this complex could provide a valuable starting point for structure-based modification of the quinoline skeleton for the development of novel antimalarials. This is of

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§ Supported by a CASE Studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) UK.
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particular interest as the quinoline family of compounds on which CQ is based are widely available, synthetically accessible, generally orally bioavailable, and of low toxicity. In combination with inhibition assays, these studies confirm the presence of a specific binding site for chloroquine in the malarial form of the enzyme, and provide guidance for modification of the quinoline structure to form an effective, selective, and competitive inhibitor for this crucial malarial enzyme.

EXPERIMENTAL PROCEDURES

**Protein Production and Crystallization**—Recombinant pfLDH and pig muscle LDH were expressed in *Escherichia coli* as described previously (1). Pig LDH is extremely similar to human LDH in sequence, structure, and activity and, because it is more readily available, was used as a representative form of mammalian LDH in this study. The fully activated forms of each enzyme were purified in a single step by affinity chromatography on an oxamate agarose column with NADH (9). Bound NADH was removed by dialysis against charcoal until the A$_{260}$/A$_{400}$ measured above 1.9.

Crystals of the apo form of pfLDH (apo-pfLDH) were obtained at 18 °C by hanging drop vapor diffusion against a well containing 20% v/v 2-methyl-2,4-pentanediol, 200 mM sodium citrate, and 100 mM HEPES, pH 7.5. The protein concentration was 50 mg ml$^{-1}$. Crystals of the pfLDH-CQ complex were obtained by soaking crystals of apo-pfLDH for 72 h in mother liquor containing 5 mM chloroquine.

**Structure Determination**—Crystals of the CQ complex belong to the space group I222 with cell dimensions of $a = 79.8$ Å, $b = 85.6$ Å, and $c = 92.2$ Å. The apo-LDH crystals have the same space group with cell dimensions of $a = 79.8$ Å, $b = 85.6$ Å, and $c = 91.8$ Å. Crystals were mounted in Hampton research cryoloops and frozen using an Oxford Cryosystems Cryostream. Crystals were frozen in mother liquor only. Data were collected on a Nonius Dif-2000 Image Plate at 100 K using Cu-K$_{α}$ radiation, filtered with mirrors, from a Nonius FR591 rotating anode generator operated at 45 kV and 95 mA. Data were processed using DENZO (10) and are summarized in Table I. The structures were solved by molecular replacement with AMORE (11) using the coordinates from the ternary complex structure of pfLDH (1). When overlaid on the ternary complex structure (Fig. 5), the quinoline ring is seen to lie in a plane similar to that of the adenyl ring of the cofactor in the ternary complex structure (1). When overlaid on the ternary complex structure (Fig. 5), the quinoline ring is seen to lie in a plane similar to that of the adenyl ring but is shifted by an average translation of 2.0 Å and rotation of about 30° in this plane. This leaves the chlorine pointing toward Asp-53, and the 4° substituent projecting toward Glu-122. The quinoline ring itself appears to be tightly bound, with average temperature factors for the ring atoms of 16.7 Å$^2$ compared with 11.3 Å$^2$ for all protein atoms. Some 471 Å$^2$ of solvent-accessible surface area is occluded between the bound CQ and the protein (calculated with QUANTA).

The drug makes a limited number of specific contacts with the protein (Fig. 4b). Most prominent is a hydrogen bond to the charged side-chain of the glutamic acid at position 122. This residue is unique to the plasmodium form of the enzyme. In all mammalian forms of LDH, and most other forms, there is a Phe in this position that is not capable of making this interaction. There are two further hydrogen bonds between CQ and the protein. The ring nitrogen is involved in a hydrogen bond network to which the side chain of Asp-53 and main chain carbonyl group and nitrogen of glycine 99 also contribute. These are both highly conserved residues found in all LDH sequences known to date and are important for cofactor binding. This suggests these contacts would not contribute any specificity for binding the malarial form of the enzyme.

Further selectivity appears to result from a series of hydrophobic interactions between the drug and enzyme. Perpendicular pi-stacking (14) between the quinoline ring system and phenylalanine side chain at position 100 provides a further interaction unique to the malarial form of the enzyme. In mammalian LDHs, the equivalent residue is always either Ala or Val, both of which are incapable of pi-stacking. This residue is at the base of the extended specificity loop, a dominant region of difference between pfLDH and all other LDH structures and

| Table I: Summary of statistics for data collection and processing and for the refined models of both the apo form of pfLDH and the CQ- pfLDH complex |
|---------------------------------|-------------------------------|
|                                  | Apo-pfLDH                     |
|                                  | CQ- pfLDH complex             |
| Resolution range                | 20–2.0 Å                      |
|                                  | 20–2.05 Å                     |
| R$_{merge}$                      | 10.1%                         |
|                                  | 6.7%                          |
| Redundancy                       | 2.6                           |
|                                  | 2.3                           |
| Completeness                     | 89.9                          |
|                                  | 99.8                          |
| Number of unique reflections    | 19,381                        |
|                                  | 20,155                        |
| Number of reflections in refinement | 18,800                     |
|                                  | 17,783                        |
| Number of waters                 | 851                           |
|                                  | 1008                          |
| Number of atoms refined (protein) | 2379                         |
|                                  | 2293                          |
| R-factor                         | 19.0%                         |
|                                  | 15.4%                         |
| R-free                           | 23.8%                         |
|                                  | 19.3%                         |
| Average B$_{iso}$ for protein atoms (Å$^2$) | 14.0                      |
|                                  | 11.3                          |
| Average B$_{iso}$ for waters (Å$^2$) | 21.1                      |
|                                  | 22.0                          |
| Overall B$_{iso}$ (Å$^2$)       | 14.8                          |
|                                  | 12.4                          |
| r.m.s. deviation for bond length (Å) | 0.007                      |
|                                  | 0.006                         |
| r.m.s. deviation for bond angles (Å) | 0.023                      |
|                                  | 0.023                         |

**RESULTS**

Both the apo and CQ complex forms of pfLDH adopt very similar overall structures (Fig. 3) with, in the absence of cofactor and substrate, characteristically open cofactor binding sites and disordered substrate specificity loops (residues 102–108). The root mean square deviation in all equivalent Ca positions between the two structures is 0.17 Å.
which forms a prominent cleft alongside the active site (1). Other nearby aromatics include Phe-52 and Tyr-85, the former again unique to pfLDH.

A dominant feature in the electron density for the quinoline is the chlorine atom. When compared with the ternary complex (Fig. 3), the chlorine is seen to occupy a similar position to the N3 atom of the adenyl ring from NADH. The chlorine makes surprisingly few contacts with the protein, these being limited to van der Waals contacts with Gly-27 and Ala-98, both of which are highly conserved residues, and limited contact with Asp-53. The bulk of this predominantly hydrophobic atom may be an important factor in orienting the quinoline for binding.

The results of the inhibition studies are consistent with chloroquine being a competitive inhibitor (with respect to NADH) of the LDH enzyme activity (Fig. 2). Analysis of the data gave CQ inhibition constants \( K_i \) of 1.3 ± 0.2 mM with pfLDH and 3.5 ± 0.3 mM with pig muscle LDH at pH 7.0. Inhibition of pfLDH at pH 8.0 by CQ is unchanged, but the \( K_i \) rises to 5 mM at pH 6.0 (data not shown). This suggests preferential binding of the monoprotonated form of CQ over the diprotonated species.

**DISCUSSION**

A comparison of the apo and CQ complex structures of pfLDH shows there is very little change in the protein structure on CQ binding (Fig. 3), with the overall root mean square deviation for positions of all Cα atoms between the two structures of only 0.17 Å. The largest changes are observed in or adjacent to the CQ binding site, where the Cα of Ile-54 moves by 0.74 Å and Asp-86 Cα by 0.8 Å. Two ordered water molecules observed in the apo structure are displaced on binding of CQ. A comparison of both the apo and CQ complex structures with the ternary structure of pfLDH (1) shows more substantial rearrangements, most significantly in the extended substrate-binding loop (residues 102–108). In the absence of bound substrate and cofactor, this loop is disordered in both the apo and CQ complex structures. This has previously been noted for apo forms of other lactate dehydrogenases (15–17).

The crystal structure of the ternary complex of pfLDH +
NADH + oxamate (a substrate analogue) revealed an unusual association between cofactor and enzyme, evidenced by an approximately 1-Å shift in the placement of the nicotinamide group relative to other forms of LDH. This alteration is believed to be an adaptation assisting the parasite to survive in anaerobic conditions prevalent within erythrocytes by reducing product inhibition associated with the enzyme. This movement originates from a series of amino acid changes along the cofactor binding groove but concentrated in the region occupied by the nicotinamide group. In the CQ binding region, there is only one minor change within van der Waals contact of the quinoline, Ile-54 (normally valine in mammalian LDHs). The overall retention of shape complementarity between the mammalian and malarial forms in this region is consistent with CQ also binding the mammalian form of the enzyme, albeit with lower affinity in the absence of the specific interactions with Asp-53, Phe-100, and Glu-122.

Chloroquine as an Inhibitor of pfLDH—Although in previous work (7) some evidence of CQ binding to pfLDH was reported, CQ was not found to inhibit pfLDH activity. The overlap of the CQ and NADH binding sites observed in the crystal structures suggested CQ should form a competitive inhibitor of the enzyme because the cofactor is required to bind and then be released during the catalytic cycle. This expectation was confirmed by the enzymatic activity experiments. The measured $K_i$ values of 1.3 and 3.5 mM for inhibition of malarial and pig LDH, respectively, are high, partially explaining previous descriptions that CQ was not inhibitory. The inhibition is competitive with respect to NADH for both enzymes, as shown by the Y-axis intercepts in Fig. 2. This behavior is consistent with the crystal structure which shows a single binding site for CQ in the NADH-adenine pocket. Although it seems unlikely that inhibition at this level would provide a sufficient explanation for the effectiveness of CQ as an antimalarial, this activity might be amplified in vivo through the high local levels of CQ known to accumulate within the parasite (3). There is some support for this in the experiments of previous workers (6, 7) where labeled CQ analogues appear to have been sufficiently concentrated within the intracellular compartments of parasites, in infected erythrocytes, to enable isolation of CQ-pfLDH affinity labeled complexes. However, the limited affinity and selectivity of CQ for the malarial form of the enzyme suggests any biological efficacy derived through this route is likely to be supplementary to other antimalarial activities of the quinolines.

The low level of inhibition by CQ correlates with the limited interactions observed between CQ and the enzyme. It is additionally unsurprising that the selectivity for the malarial form of the enzyme is not pronounced. Despite the specific interactions described above, there remain a significant number of common structural features within the CQ binding site between the malarial and pig LDH structures. However, although the $K_i$ values are relatively high, it is important to note that the association of CQ with the malarial form is favored by a net change in free energy equivalent to about 2 kJ/mol relative to mammalian LDH. This modest gain suggests that the quinoline template might provide a useful lead from which further specificity and binding could be developed. As glycolysis is the principle source of ATP for the parasite, which cannot undertake oxidative phosphorylation (18), disruption of the glycolytic pathway by inhibition of pfLDH appears to be an effective route for killing the parasite. The proximity of the CQ binding site to other regions of the enzyme both important for its activity and of unique structure compared with human LDH implies that the inclusion of quinoline moieties in composite compounds capable of bridging these regions might lead to dramatic additive increases in both selectivity and inhibitory activity. Parallels for the development of inhibitors to nucleotide binding sites have, for example, recently been described by Gray et al. (19) where a series of kinase inhibitors have been synthesized based on the binding of substituted purines to the ATP binding site of human cyclin-dependent kinase2 (CDK2).

Menting et al. (7) have previously reported that pfLDH is particularly sensitive to inhibition by hematin and that CQ reduces this activity by 2 orders of magnitude. Using fluorescence studies, we have been able to show hematin binds tightly to pfLDH ($K_d = 0.25 \mu M$, results not shown) but so far have been unable to produce crystals of a hematin-pfLDH complex. The modulatory effect of CQ on hematin inhibition is consistent with related binding sites for both compounds, and the CQ/hematin adduct, on the enzyme.

Conclusions—The mode of action of chloroquine and related quinolines has remained unclear despite extensive usage of these compounds as antimalarials for many years. Structural analysis of complexes of these compounds with their molecular targets could provide an invaluable boost to the design of new drugs urgently needed to overcome developing drug resistance. Previous studies (6, 7) identified pfLDH as a potential molecular target for CQ. In this study we have shown that CQ acts as a modest competitive inhibitor of the enzyme and have determined the first structure of a complex between chloroquine and an enzyme. Although we remain cautious of the importance of pfLDH inhibition in the overall efficacy of CQ, the structure of this complex demonstrates that specific targeting of this important glycolytic enzyme from the parasite should be feasible. Structure-based drug design studies incorporating this template could lead to the production of new forms of efficient pfLDH inhibitors as antimalarials.

Acknowledgments—We are grateful to Mark Banfield, for general assistance with pfLDH preparation and crystallography, and to Christopher Higham, Dilek Turgut-Balik, and John Holbrook, for access to the pfLDH clone.
REFERENCES

1. Dunn, C. R., Banfield, M. J., Barker, J. J., Higham, C. W., Moreton, K. M., Turgut-Balik, D., Brady, R. L., and Holbrook, J. J. (1996) Nat. Struct. Biol. 3, 912–915
2. Peters, W. (1970) Chemotherapy and Drug Resistance in Malaria, pp. 549–601, Academic Press, London
3. Yayon, A., Cabantchick, Z. I., and Ginsburg, H. (1984) EMBO J. 3, 2695–2700
4. Fitch, C. D. (1972) Proc. Helminthol. Soc. Washington 39, 265–271
5. Ginsburg, H., and Geary, T. G. (1987) Biochem. Pharmacol. 36, 1567–1576
6. Foley, M., Deady, L. W., Ng, K., Cowman, A. F., and Tilley, L. (1994) J. Biol. Chem. 269, 6955–6961
7. Menting, J. G. T., Tilley, L., Deady, L. W., Ng, K., Simpson, R. J., Cowman, A. F., and Foley, M. (1997) Mol. Biochem. Parasitol. 88, 215–224
8. Chou, A. C., Chevli, R., and Fitch, C. D. (1980) Biochemistry 19, 1543–1549
9. Wilks, H. M., Moreton, K. M., Halsall, D. J., Hart, K. W., Sessions, K. D., Clarke, A. R., Holbrook, J. J. (1992) Biochemistry 31, 7802–7806
10. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
11. Bailey, S. (1994) Acta Crystallogr. Sec. D 50, 760–763
12. Brünger, A. T. (1992) X-PLOR Version 3.1, Yale University Press, New Haven, CT
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Haven, CT

13. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sec. A 47, 110–119
14. Singh J., and Thornton J. M. (1985) FEBS Lett. 191, 1–6
15. White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Lenz, P. J., Smiley, I. E., Steindel, S. J., and Rossmann, M. G. (1976) J. Mol. Biol. 102, 759–779
16. Musick, W. D. L., and Rossmann, M. G. (1979) J. Biol. Chem. 254, 7611–7620
17. Piontek, K., Chakrabarti, P., Schar, H. P., Rossmann, M. G., and Zaber, H. (1990) Proteins Struct. Funct. Genet. 7, 74–92
18. Vander Jagt, D. L., Hunsaker, L. A., Campos, N. M., and Baack, B. R. (1990) Mol. Biochem. Parasitol. 42, 277–284
19. Gray, N. S., Wodicka, L., Thunnissen, A.-M. W. H., Norman, T. C., Kwon, S., Espinoza, F. H., Morgan, D. O., Barnes, G., LeClerc, S., Meijer, L., Kim, S.-H., Lockhart, D. J., and Scholtz, P. G. (1998) Science 281, 533–538
20. Brunger, A. T. (1992) Nature 355, 472–474