Proc. Nat. Acad. Sci. USA
Vol. 72, No. 8, pp. 3057–3059, August 1975
Biochemistry

On primary structure and biosynthesis of histidine-rich polypeptide from malarial parasite *Plasmodium lophurae*

(puromycin)

ARAXIE KILEJIAN, TA-HSIU LIAO*, AND WILLIAM TRAGER

The Rockefeller University, New York, N.Y. 10021

Contributed by William Trager, June 5, 1975

ABSTRACT **Experiments on the primary structure of a histidine-rich polypeptide isolated from the malarial parasite *Plasmodium lophurae* indicate that the smaller quantities of amino acids other than histidine form an integral part of the polypeptide and do not arise from a protein contaminating a histidine homopolymer. In culture, the parasites incorporate over 50% of exogenously supplied histidine into the histidine-rich polypeptide and this incorporation is inhibited by puromycin.**

The intraerythrocytic stages of the avian malarial parasite *Plasmodium lophurae* contain numerous cytoplasmic granules (1). The main chemical constituent of isolated granules was shown to migrate as a single polypeptide on polyacrylamide gels, and analysis of hydrolysates of the polypeptide showed five major constituent amino acids: 73% histidine, 7.5% proline, 7% alanine, 6% glutamic acid, and 2% aspartic acid (2). The unusually high content of histidine raised two questions: *(a)* could the smaller quantities of other amino acids come from a typical protein isolated as a contaminant with the histidine homopolymer rather than being integral parts of the polypeptide, and *(b)* could the polypeptide possibly be synthesized by a non-ribosomal enzymatic system (3–5). Even though non-ribosomal synthesis of peptides has been shown only in prokaryotes, the possibility that it might also exist in eukaryotes cannot be excluded. The unusually high content of one amino acid, histidine, and some parallelism with the polypeptide of the cyanophycin granules of blue-green algae made the malaria polypeptide a plausible candidate for testing. The algal polypeptide was shown to be a high-molecular-weight copolymer of argine and aspartic acid, and its synthesis was not inhibited by chloramphenicol (5, 6).

To answer the above questions, we undertook experiments on the primary structure of the peptide and studied the effect of puromycin on the biosynthesis of the peptide.

MATERIALS AND METHODS

Materials. The histidine-rich polypeptide was isolated as previously described (2). Pepsin, trypsin, and chymotrypsin were purchased from Worthington; Nagarse was from Enzyme Development Corp., New York, and puromycin from Calbiochem. L-[^{3}H]Histidine, (general label, specific activity 3.1 Ci/mmol), was purchased from Schwarz/Mann. Sephadex G-50 was from Pharmacia. All other chemicals were reagent grade.

Enzymatic Hydrolysis of the Histidine-Rich Polypeptide. Two hundred and sixty μg of the polypeptide were digested for 2 hr at 37° by pepsin (5 μg, in 0.0005 N HCl), trypsin (20 μg, in 0.25% NH_4HCO_3), chymotrypsin (20 μg, in 0.25% NH_4HCO_3), or Nagarse (20 μg, in 0.25% NH_4HCO_3) in a volume of 50 μl, and the digests were spotted on paper and subjected to electrophoretic analysis. In the preparative scale, 13.3 mg (0.35 μmol) of the polypeptide were hydrolyzed by 0.5 mg of Nagarse for 2 hr at 37° in 2 ml of 0.25% NH_4HCO_3, and the digest was gel filtered on a column of Sephadex G-50.

Paper Electrophoresis. Paper electrophoresis was carried out in tanks (Savant) on Whatman no. 3 MM paper. The buffer system used was pyridine–acetic acid–water (100:4:900), pH 6.5. The sample was applied at the middle of the strip. For preparative purposes, guide strips were cut and stained with ninhydrin–cadmium reagent (7) and peptides were eluted with 30% acetic acid.

Amino-Acid Analysis. Peptides were hydrolyzed in 200 μl of 6 N HCl containing 0.2% (w/v) phenol and 0.1% (v/v) mercaptoacetic acid in evacuated sealed tubes, at 110° for 22 hr (8). Hydrolysates were analyzed with a modified amino-acid analyzer (9).

Polyacrylamide Gel Electrophoresis. Electrophoresis was according to the method of Panyim and Chalkley (10).

Incorporation of [^{3}H]Histidine *In Vitro*. Uninucleate trophozoites of *P. lophurae* were removed from their host duck erythrocytes and cultured extracellularly under conditions described previously (11). To facilitate quantitative collection of parasites at the end of the incubation period, the plasma clot lining of the culture flask was omitted. To each of 3.5 ml medium in eight culture flasks 50 μCi of L-[^{3}H]histidine was added. To four of these flasks puromycin was also added to give a final concentration of 20 μg/ml. At the end of a 4 hr incubation period, the contents of each flask were transferred quantitatively to centrifuge tubes with three 5 ml buffer (12) rinses. Parasites were pelleted and washed once with buffer. Duplicate parasite pellets were assayed for radioactivity incorporated into total protein and the histidine-rich polypeptide. As a measure of total protein synthesis, radioactivity in cold 10% trichloroacetic acid insoluble pellet was determined after solubilization and decolorization of the pellet (13). To measure synthesis of the histidine-rich polypeptide, parasite pellets were extracted with 5% acetic acid, and extracts were evaporated to dryness under vacuum. The dry residue was dissolved in 200 μl of 5% acetic acid–5 M urea to which purified histidine-rich polypeptide was added as a carrier and measured aliquots were applied to polyacrylamide gels. After destaining, gels were sliced into 0.5 cm slices and solubilized with 0.3 ml of

* Present address, Department of Biochemistry, Oklahoma State University, Stillwater, Okla. 74074

Downloaded by guest on April 17, 2021

Table 1. Amino-acid compositions of histidine-rich polypeptide and peptide fractions from gel-filtration of the Nagarse digest

Amino acid	Histidine-rich polypeptide	Fraction A	Fraction B	Fraction C	Fraction D
	Mole percent				
Asp	2.61	1.01	0.99	6.21	2.97
Thr	0.30	0.36		0.07	1.50
Ser		0.61*			0.55*
Glu	6.41	8.29	8.09	4.83	7.57
Pro	8.18	13.34	10.73	2.99	6.64
Gly	0.84	1.17	2.35	1.03	2.96
Ala	6.79	6.52	8.16	11.02	3.35
Val	0.33	0.80			4.14
Ile		0.39*			0.51*
Leu	1.05	0.70	0.37	0.63	4.46
Tyr		0.46*			0.55*
Phe	1.29				4.73
His	72.49	65.83	69.28	73.19	59.87
Lys		0.45*			0.13*

* These amino acids which are not present in the starting material are probably derived from the added Nagarse.

30% H_2O_2 (4 hr at 60°), counting fluid (Aquasol, New England Nuclear) was added, and radioactivity was determined.

RESULTS AND DISCUSSION

Composition of peptides

In the preliminary experiment, extensive proteolysis of the histidine-rich peptide was observed only with Nagarse; hydrolysis with pepsin, trypsin, or chymotrypsin was either ineffective or insufficient. The conclusions were based upon electrophoretic patterns obtained with the hydrolysates. Nagarse was therefore chosen for the subsequent studies.

The digest of the polypeptide with Nagarse was first gel-filtered through a column of Sephadex G-50 in 50% acetic acid. The elution pattern, analyzed with ninhydrin after alkaline hydrolysis (14), is shown in Fig. 1. Amino-acid analysis of each of the first four fractions (A, B, C, and D) showed that histidine was still the major constituent amino acid (Table 1). Polyacrylamide gel electrophoresis of three of the fractions as well as the whole digest showed the absence of the intact polypeptide chain (Fig. 2). Fraction D, which contained small oligopeptides, was further fractionated by paper electrophoresis. Seven fractions were separated distinctively. The fractions were numbered according to their migration in the electrical field; Fraction 1 was the most negatively charged. Amino-acid analyses of six of the seven fractions are shown in Table 2 (one of the fractions was lost during the preparation). As judged from the composition, some fractions contain mostly a single peptide. This is especially noted in Fractions 1 and 2 in which both histidine and glutamic acid are present. Since electrophoresis was carried out at pH 6.5, any histidine detected in a peptide that moves toward the anode must be an integral part of the peptide. If the neutral and acidic amino acids were to originate from a typical protein adsorbed to polyhistidine, neutral or acidic histidine-free peptides would be expected; none were observed. Good recovery of the two peptides (Fraction D 1 yielded 42.9% and Fraction D 2 yielded 42.0%) strongly supports the molecular weight calculations of the intact polypeptide and the conclusion that the polypeptide contains amino acids other than histidine.

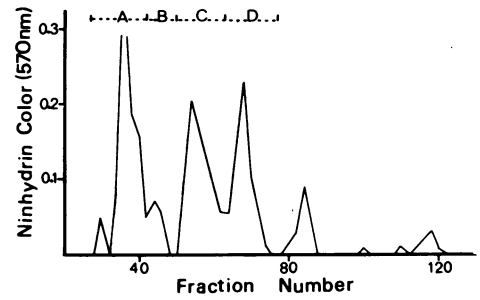

FIG. 1. Separation of peptides. The polypeptide (0.35 μmol) was hydrolyzed by 0.5 mg of Nagarse for 2 hr at 37° and the digest was gel-filtered on a 2.5 × 160 cm column of Sephadex G-50 in 50% acetic acid. Fractions of 4 ml were collected and 0.1 ml aliquots from every second tube were analyzed with ninhydrin after alkaline hydrolysis.

Table 2. Amino-acid composition of peptide fractions isolated from Fraction D of Nagarse digest of histidine-rich polypeptide by paper electrophoresis

Amino Acid	Fraction D1	Fraction D2	Fraction D3	Fraction D4	Fraction D5	Fraction D7
	Residues per mole		Mole percent			
Asp	1.07(1)	0.24	7.97	1.57	2.32	
Thr	0.95(1)	0.05	3.73	0.32		
Ser		0.15	9.99	0.63		
Glu	2.02(2)	2.15(2)	7.54	0.57		
Pro	0.91(1)		9.20	19.69		
Gly	0.04	0.26	16.67	1.53	10.90	1.45
Ala	0.01	0.08	11.50	2.82	5.10	1.24
Val	2.09(2)		9.27			
Ile			7.54	0.18		
Leu		0.95(1)	9.05	5.42	14.90	
Tyr					7.50	6.06
His	0.95(1)	1.89(2)	7.47	67.24	59.24	91.23

The numbers in parentheses are the possible number of residues in a peptide.

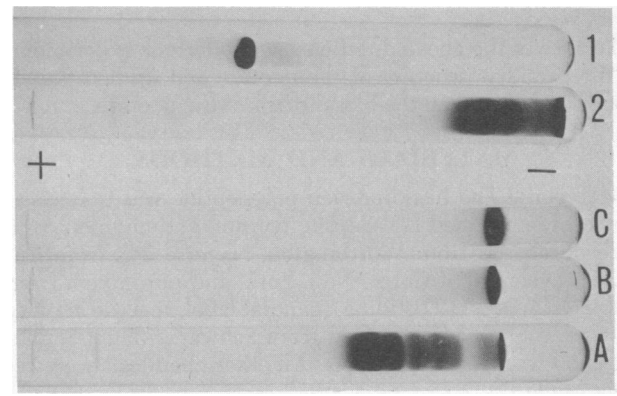

FIG. 2. Electrophoresis in 15% polyacrylamide gel at pH 3.2 in 6.25 M urea. 1, histidine-rich polypeptide used for analysis; 2, total Nagarse digest shows the absence of intact polypeptide chain; A, B and C are the fractions shown in Fig. 1. Gels 1 and 2 were from a different electrophoretic run (running time 4 hr) than gels A, B, and C (running time 3 hr)

Downloaded by guest on April 17, 2021

Table 3. Effect of puromycin on the incorporation of [^{3}H]histidine into total protein and the histidine-rich polypeptide

Sample	Control	Puromycin added
	(cpm per 70 × 10^6 parasites)	
Total protein	58,470	15,470
	66,720	19,740
Histidine-rich polypeptide	32,040	3,030
	38,110	3,400

Each pair of values represents duplicate samples.

Incorporation of [^{3}H]histidine

The addition of puromycin to culture media inhibited significantly incorporation of labeled histidine into total protein as well as the histidine-rich polypeptide (Table 3). Results are expressed in cpm since neither the specific activity of histidine in the incubation medium nor in the parasite amino-acid pool were known. It is of interest that 50% of total incorporated label is recovered in the histidine-rich polypeptide; this is an underestimation when reduced efficiency of detection of radioactivity in gel slices is considered.

Since uninucleate parasites are at a stage of development that shows rapid synthesis of the polypeptide, the assumption has been made that the enzyme systems required for its synthesis are present and that the observed inhibition by puromycin suggests the ribosomal route for the synthesis of the polypeptide. At the end of the incubation period in the presence of puromycin, parasites appeared structurally normal by phase contrast microscopy.

The results of the structural as well as the incorporation studies indicate that the unusual histidine-rich polymer of *P. lophurae* is a protein and not simply a reserve material synthesized non-ribosomally, as is the polypeptide of the cyanophycin granules observed in algae.

We thank Dr. Stanford Moore for helpful discussions and suggestions. This work was supported in part by U.S. Public Health Service Grants AI-10640 and GMO7256.

1. Hewitt, R. (1942) "Studies on the host-parasite relationships of untreated infections with *Plasmodium lophurae* in ducks," *Amer. J. Hyg.* **36**, 6–40.
2. Kilejian, A. (1974) "A unique histidine-rich polypeptide from the malaria parasite, *Plasmodium lophurae*," *J. Biol. Chem.* **249**, 4650–4655.
3. Lipmann, F., Jevers, W., Kleinkauf, H. & Roskoski, R. (1971) "Polypeptide synthesis on protein templates: the enzymatic synthesis of gramicidin S and tyrocidine," in *Advances in Enzymology*, ed. Meister, A. (John Wiley & Sons, New York), Vol. 35, pp. 1–34.
4. Kamiryo, T. & Matsuhashi, M. (1972) "The biosynthesis of the cross-linking peptides in the cell wall peptidoglycan of *Staphylococcus aureus*," *J. Biol. Chem.* **247**, 6306–6311.
5. Simon, R. D. (1973) "The effect of chloramphenicol on the production of cyanophycin granule polypeptide in the blue-green alga *Anabaena cylindrica*," *Arch. Mikrobiol.* **92**, 115–122.
6. Simon, R. D. (1971) "Cyanophycin granules from the blue-green alga *Anabaena cylindrica*: A reserve material consisting of copolymers of aspartic acid and arginine," *Proc. Nat. Acad. Sci. USA* **68**, 265–267.
7. Dreyer, H. N. & Bynum, E. (1967) "High-voltage paper electrophoresis," in *Methods in Enzymology*, ed. Hirs, C. H. W. (Academic Press, New York), Vol. XI, pp. 32–39.
8. Moore, S. & Stein, W. H. (1963) "Chromatographic determination of amino acids by the use of automatic recording equipment," in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. D. (Academic Press, New York), Vol. VI, pp. 819–831.
9. Liao, T.-H., Robinson, G. W. & Salnikow, J. (1973) "Use of narrow-bore columns in amino acid analysis," *Anal. Chem.* **45**, 2286–2288.
10. Panyim, S. & Chalkley, R. (1969) "High resolution acrylamide gel electrophoresis of histones," *Arch. Biochem. Biophys.* **130**, 337–346.
11. Trager, W. (1971) "Malaria parasites *(Plasmodium lophurae)* developing extracellularly *in vitro*: incorporation of labeled precursors," *J. Protozool.* **18**, 392–399.
12. Trager, W. (1959) "The enhanced folic and folinic acid contents of erythrocytes infected with malaria parasites," *Exp. Parasit.* **8**, 265–273.
13. Mahin, D. T. & Lofberg, R. T. (1966) "A simplified method of sample preparation for determination of tritium carbon-14 or sulfur-35 in blood or tissue by liquid scintillation counting," *Anal. Biochem.* **16**, 500–509.
14. Moore, S. (1968) "Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction," *J. Biol. Chem.* **243**, 6281–6283.

Downloaded by guest on April 17, 2021