Arch Microbiol (1984) 137:104–108

Archives of
Microbiology

Effects of chloroquine on proteolytic processes and energy metabolism in yeast

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Abstract. In yeast cells, degradation of cellular proteins was inhibited by addition of chloroquine to the medium as shown by a decrease of the release of trichloroacetic acid-soluble radioactivity from prelabelled cell protein. Penetration of chloroquine into the cells was strongly enhanced with increasing pH value of the medium. The concentration in the cells reached 5–14 times that in the medium of pH 8.0. Fluorescence microscopy showed that chloroquine was concentrated in the vacuoles of the cells. Chloroquine, at concentrations attained in the cells, inhibited the activities of vacuolar proteinases in vitro. Furthermore, chloroquine caused a rapid and drastic decrease of the ATP content of the cells and prevented the fermentation of glucose and formation of ethanol under aerobic conditions.

Key words: Chloroquine – Yeast – Proteolysis – ATP hydrolysis – Glucose consumption – Ethanol formation

In the course of our investigations on intracellular proteolysis in yeast (Holzer 1976; Holzer and Heinrich 1980; Müller and Holzer 1981a), the effect of chloroquine, known as an inhibitor of lysosomal proteolytic processes in mammalian cells (Wibo and Poole 1974), was studied. Chloroquine was found to inhibit glucose-induced catabolite inactivation of cytoplasmic malate dehydrogenase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (Lenz 1980; Müller and Holzer 1981a). A proteolytic mechanism of catabolite inactivation was shown for cytoplasmic malate dehydrogenase by Neeff et al. (1978), for fructose-1,6-bisphosphatase by Funayama et al. (1980) and for phosphoenolpyruvate carboxykinase by Müller et al. (1981). The inhibitory effect of chloroquine on catabolite inactivation of phosphoenolpyruvate carboxykinase and cytoplasmic malate dehydrogenase (Lenz 1980; Müller and Holzer 1981a) is consistent with the proteolytic mechanism. Catabolite inactivation of fructose-1,6-bisphosphatase, however, was less sensitive towards inhibition by chloroquine than inactivations of phosphoenolpyruvate carboxykinase and cytoplasmic malate dehydrogenase (Lenz 1980; Müller and Holzer 1981a). This unexpected finding prompted detailed studies on the mechanism of glucose-induced catabolite inactivation of fructose-1,6-bisphosphatase (Lenz and Holzer 1980; Holzer et al. 1981; Tortora et al. 1981) and led to the discovery of phosphorylation of the enzyme (Müller and Holzer 1981b; Mazón et al. 1982) preceding its proteolytic degradation.

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Since chloroquine has been increasingly used as a tool for investigating intracellular proteolysis in yeast, studies on the action of chloroquine on yeast cells were performed. It is shown in the present paper that the penetration of chloroquine into the yeast cells was strongly pH-dependent and that chloroquine was accumulated in the vacuoles of the cells. Degradation of cellular proteins in intact cells was inhibited by addition of chloroquine as shown in experiments with cells pregrown in the presence of labelled leucine. Inhibition of certain vacuolar proteinases by chloroquine was observed using cell-free extracts. In addition, effects of chloroquine on the concentration of ATP and on carbohydrate metabolism in yeast were observed which cannot be explained as a consequence of inhibition of vacuolar proteolysis.

Materials and methods

Materials

The reagents for the enzyme assays and the ATP-Biolumineszenz-CLS test combination for determination of ATP were purchased from Boehringer (Mannheim, FRG). Azocoll was obtained from Calbiochem (Gießen, FRG), L-leucine-p-nitroanilide from Bachem (Buchendorf, Switzerland) and N-benzoyl-L-tyrosine-p-nitroanilide from Serva (Heidelberg, FRG). L-[4,5-^{3}H]leucine was obtained from Amersham Buchler (Braunschweig, FRG). Toluene Scintillator was from Roth (Karlsruhe, FRG). All yeast growth media were purchased from Difco (Schmid, München, FRG). Chloroquine and L-malic acid were from Sigma (Taufkirchen, FRG). Hemoglobin and all other chemicals, which were of highest purity available, were obtained from Merck (Darmstadt, FRG).

Yeast strain and growth conditions

The diploid yeast *Saccharomyces cerevisiae* strain M_1 (supplied by Dr. A. W. Linnane, Monash University, Clayton, Australia) was used throughout this study. Growth conditions, harvest of cells and preparations of crude extracts were carried out as previously described (Lenz and Holzer, 1980).

Enzyme assays

Phosphoenolpyruvate carboxykinase (Hansen et al. 1976), malate dehydrogenase (Wolfe and Neilands 1956) and fructose-1,6-bisphosphatase (Gancedo and Gancedo 1971) were assayed according to the published procedures. Proteinase A was measured against acid-denatured hemoglobin at pH 3 as described by Saheki and Holzer (1974).

Proteinase B was determined according to Saheki and Holzer (1974) using Azocoll as substrate. Carboxypeptidase Y was assayed as reported by Aibara et al. (1971) with N-benzoyl-L-tyrosine-p-nitroanilide as substrate. Vacuolar aminopeptidase I activity was tested with L-leucine-p-nitroanilide as substrate according to Frey and Röhm (1978). The enzymatic determinations of glucose and ethanol were done as described by Bergmeyer et al. (1974) and Bernt and Gutmann (1974), respectively.

Determination of chloroquine

Chloroquine was determined by measuring the optical density in 15 mM phosphate buffer at pH 5.9 and 343 nm. The observed values were corrected by substracting the optical density of control cell extracts, which were not incubated with chloroquine.

Flurorescence microscopy

Fluorescence was observed with a standard Zeiss fluorescence microscope with exiter filter BP 365/11, a color splitting filter FT395, a barrier filter LP 397 and a 100 × oil-immersionphase objective. Photographs were taken with a black and white negative film with variable film speed (Agfapan Vario XL) at 5 min exposures. Chloroquine, excited with light of 330 nm, exhibited maximal fluorescence at 390 nm (Stollar and Levine 1963).

Determination of protein degradation

Cellular proteins were prelabelled with L-[^{3}H]leucine during growth in a minimal medium [0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) glucose] for 48 h. After washing twice with sterile water, cells were transferred into the respective medium containing 2 mM non-radioactive leucine as chase. Degradation of proteins was measured by following the release of trichloroacetic acid-soluble radioactivity from the prelabelled protein as described by Betz and Weiser (1976). Protein was solubilized with 1 ml NCS/H_2O (9/1, v/v). Radioactivity was counted in 10 ml Triton X-100/Toluene (1/2, v/v) scintillation mixture (Patterson and Green 1965). Different quenching of protein-containing samples and trichloroacetic acid supernatants were corrected by internal standardization.

Determination of ATP

At the times indicated, 0.1 ml aliquots of the incubation mixtures were added to the same volume of ice-cold 35% (v/v) perchloric acid. After 20 min incubation at 0°C with occasional shaking, the extraction procedure was stopped by adding 0.54 ml of 2 M potassium hydrogen carbonate to get a final pH value of about 7.0. After 10 min standing at 0°C, the perchlorate formed was separated by centrifugation for 4 min at 8,000 × *g*. The supernatants were diluted 1 to 200 with quartz-destilled water. Standard ATP solutions were added for calibration to a final concentration of 5×10^{-9} M ATP. To 0.2-ml aliquots of these mixtures, 0.2-ml of luciferase/luciferin reagent-solution were added and the luminescence was measured in a Biolumat LB 9500 T (Berthold, Wildbad, FRG).

Determination of protein

Protein was estimated according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Table 1. Influence of chloroquine on catabolite inactivation. Yeast cells from stationary growth phase (2% wet weight/volume) were suspended in 0.1 M potassium phosphate buffer, pH 7.4, at 30°C. Aliquots of this cell suspension were incubated in the presence or absence of 2% (w/v) glucose with the indicated concentrations of chloroquine. At zero time and after two hours of incubation, 2 ml samples were withdrawn and centrifuged at 4°C at about 8,000 × *g* for 4 min. Preparations of crude extracts and enzyme assays were performed as described under Materials and methods. Values in the table represent catabolite inactivation after two hours of incubation. Catabolite inactivation was defined as

$$\text{C.I.} = \frac{(\text{activity without glucose}) - (\text{activity with glucose})}{(\text{activity without glucose})} \times 100$$

	% Catabolite inactivation					
Concentration of chloroquine (mM)	0	5	10	20	50	100
Phosphoenolpyruvate carboxykinase	81	78	<5	13	<5	<5
Malate dehydrogenase	37	42	16	17	<5	<5
Fructose-1,6-bisphosphatase	96	96	88	69	47	35

Table 2. Effect of pH of the medium on the inhibition of catabolite inactivation caused by 10 mM chloroquine. For experimental conditions and definition of catabolite inactivation, see legend to Table 1. The levels of the catabolite inactivation in the absence of chloroquine at all pH values were 76% for phosphoenolpyruvate carboxykinase, 50% for malate dehydrogenase and 97% for fructose-1,6-bisphosphatase, respectively

	% Catabolite inactivation				
pH	6.0	6.5	7.0	7.5	8.0
Phosphoenolpyruvate carboxykinase	69	68	57	<5	<5
Malate dehydrogenase	49	40	46	<5	<5
Fructose-1,6-bisphosphatase	97	97	90	61	15

Results and discussion

The influence of chloroquine on glucose-induced catabolite inactivation of phosphoenolpyruvate carboxykinase, cytoplasmic malate dehydrogenase and fructose-1,6-bisphosphatase is shown in Table 1. The inactivation of phosphoenolpyruvate carboxykinase and cytoplasmic malate dehydrogenase was inhibited by chloroquine to almost the same extent whereas the inactivation of fructose-1,6-bisphosphatase was less sensitive. As can be seen from Table 2, the inhibition of catabolite inactivation caused by chloroquine was pH-dependent. The inhibitory effect of chloroquine on the inactivation of all three enzymes enhanced with increasing pH value of the medium. This result points to penetration of the undissociated form of chloroquine into the cell as it exists in the more alkaline pH range, followed by intracellular protonisation and accumulation. Such a mechanism was previously proposed by de Duve et al. (1974) for the accumulation of chloroquine in lysosomes of mammalian cells. Table 3 shows the pH-dependency of the accumulation of chloroquine in yeast cells. Raising the pH value of the incubation medium from 6.0 to 8.0 caused a 14-fold increase of the intracellular concentration of chloroquine when incubated in the presence of glucose. In the absence of glucose,

Table 3. Intracellular and extracellular concentrations of chloroquine. Cells from stationary growth phase (2% wet weight/volume) were suspended in 0.1 M potassium phosphate buffer of the indicated pH and were incubated with and without 2% (w/v) glucose and 10 mM chloroquine. The concentration of chloroquine after two hours of incubation was determined in the supernatant of centrifuged samples and in the crude extract of cells as described in Materials and methods

	A chloroquine/ml supernatant (μMol/ml)		B chloroquine/g yeast (wet weight) (μMol/g)		B/A accumulation (-fold)	
pH	−glucose	+glucose	−glucose	+glucose	−glucose	+glucose
6.0	8.2	8.1	7.1	9.8	1	1
6.5	8.0	7.8	17	14	2	2
7.0	8.2	7.6	26	31	3	4
7.5	7.9	7.6	34	72	4	9
8.0	7.7	6.9	40	96	5	14

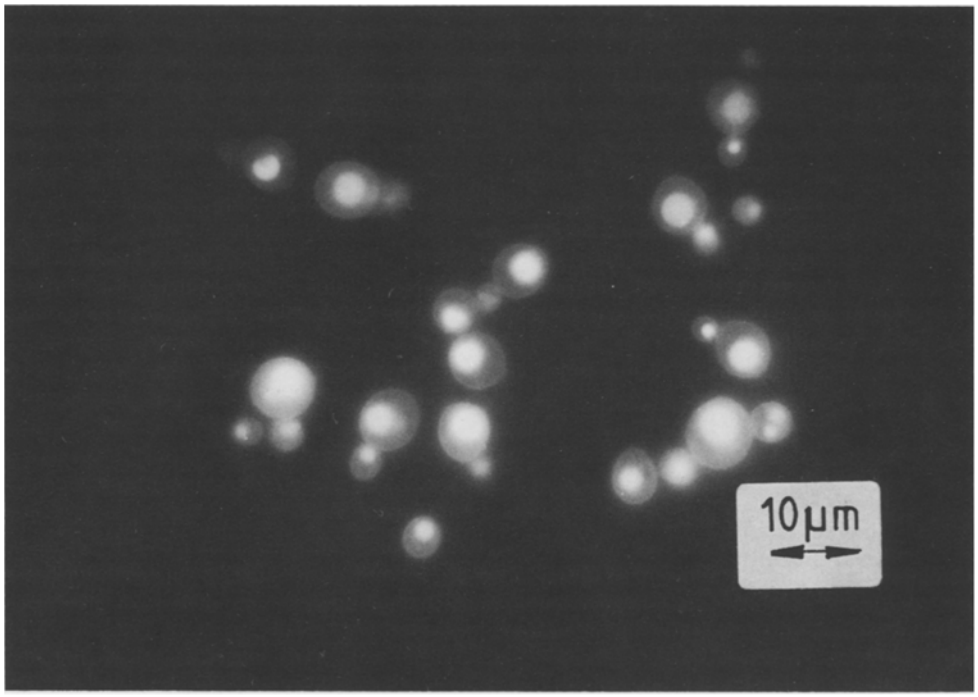

Fig. 1
Fluorescence microscope photograph of yeast cells from stationary growth phase incubated for 30 min with 2% (w/v) glucose and 10 mM chloroquine. The procedure is outlined in Materials and methods

the intracellular accumulation of chloroquine was significantly lower. Different intracellular pH values during incubation with and without glucose might be responsible for the difference. The chloroquine accumulation in the cells could be detected by fluorescence microscopy of glucose incubated cells as shown in Fig. 1. There was a moderate fluorescence corresponding to chloroquine equally distributed in the whole cell. The intracellular particles characterized by an intensive fluorescence corresponded to what is known from other microscopical studies to be vacuoles (Wiemken et al. 1979). Cells incubated in the absence of glucose showed a different fluorescence pattern as compared to those incubated in the presence of glucose. This difference will be further investigated. In Fig. 2, the effect of chloroquine on extracted vacuolar proteinase of yeast is shown. The assays were done at pH 3.0 for proteinase A, at pH 7.0 for proteinase B and carboxypeptidase Y, and at pH 7.5 for aminopeptidases. Hydrolysis of leucine-p-nitroanilide, used as the substrate for the determination of aminopeptidases, indicates the combined activities of the aminopeptidases I and II (Frey and Röhm 1978). A distinct inhibition of proteolytic activities by 10 to 20 mM chloroquine was observed at the respective pH values. Similar to the studies of Wibo and Poole (1974) and Ohkuma and Poole (1978) on the action of chloroquine, NH_3 and other weak bases on mammalian lysosomes, our findings indicate effects of chloroquine on proteolysis which are not simply the consequence of an increase of the pH value in the vacuoles of yeast cells. The release of trichloroacetic acid-soluble radioactivity was measured as a parameter for proteolysis in intact yeast cells, labelled during growth on a [^{3}H]-leucine-containing medium. It may be seen from Fig. 3 that 10 mM chloroquine at pH 7.5 significantly inhibited proteolysis. The incubation with 20 mM chloroquine for three hours completely prevented proteolytic release of trichloroacetic acid-soluble radioactivity.

In order to investigate the specificity of the chloroquine effect on proteolysis, ATP was assayed in cells treated with chloroquine. As shown in Fig. 4a, 20 mM chloroquine in the presence of glucose decreased the steady state concentration of ATP to about one half in 20–30 min; chloroquine at

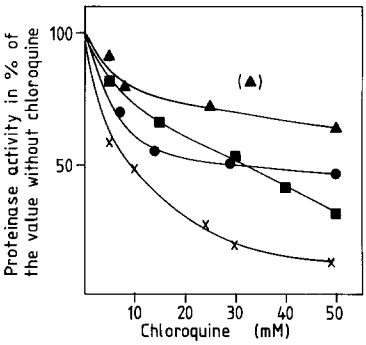

Fig. 2. Inhibition of yeast proteinase activities by chloroquine. Partially purified yeast proteinase A (Meussdoerffer et al. 1980), proteinase B (Betz et al. 1974), carboxypeptidase Y (Jušić et al. 1976) and a crude yeast extract containing aminopeptidases were incubated with the indicated concentrations of chloroquine. The enzyme activities were measured as described under Materials and methods. (▲——▲) Proteinase A; (■——■) proteinase B; (●——●) carboxypeptidase Y; (×——×) aminopeptidases I and II

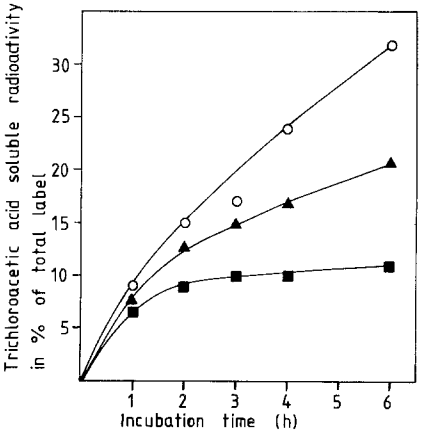

Fig. 3. Degradation of cellular proteins in the presence of chloroquine. Stationary cells grown on L-[^{3}H]-leucine-containing medium were washed and transferred into 0.1 M potassium phosphate buffer, pH 7.5, containing 2 mM non-radioactive leucine, 2% (w/v) glucose (○——○), and additionally 10 mM chloroquine (▲——▲) or 20 mM chloroquine (■——■). Incubation was performed at 30° C. 1 ml samples were taken at the times indicated and the trichloroacetic acid-soluble radioactivity was determined as outlined under Materials and methods

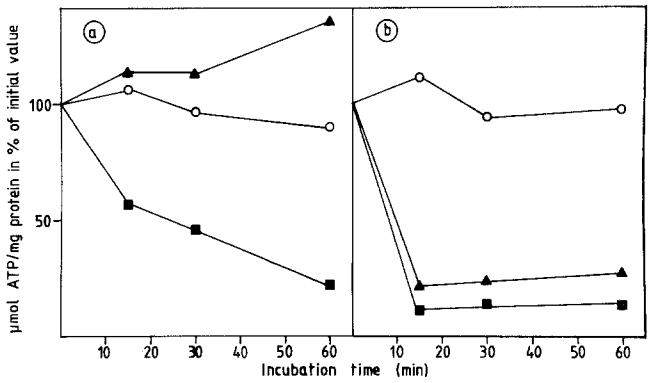

Fig. 4a and b. Effect of chloroquine on the ATP content of cells. Cells were incubated in the presence **a** or absence **b** of 2% (w/v) glucose at 30° C and pH 7.5. Without addition of chloroquine (○——○); with addition of 10 mM chloroquine (▲——▲); or 20 mM chloroquine (■——■). Samples of 0.1 ml were taken at times indicated and assayed for ATP as described in Materials and methods. The initial ATP concentration in the presence of glucose was 0.013 µmol ATP/mg protein or 1.12 µmol ATP/g wet weight and, in the absence of glucose, 0.011 µmol ATP/mg protein or 0.93 µmol ATP/g wet weight

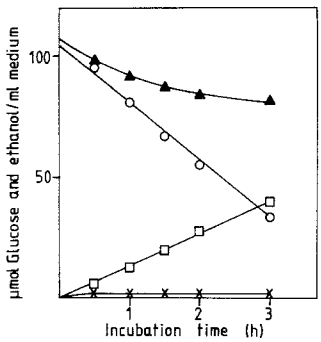

Fig. 5. Effect of chloroquine on aerobic glucose consumption and alcoholic fermentation. Cells were incubated under aerobic conditions with 2% (w/v) glucose at pH 7.5 and 30° C. Samples were collected and centrifuged at times indicated and the concentrations of glucose and ethanol were determined in the supernatant as described in Materials and methods. Glucose consumption without addition of chloroquine (○——○); with addition of 10 mM chloroquine (▲——▲). Ethanol formation without addition of chloroquine (□——□); with addition of 10 mM chloroquine (×——×)

10 mM was ineffective. In the absence of glucose, the effect of chloroquine was much more marked. Chloroquine, even at 10 mM, diminished the ATP level to about 20% of the initial value in less than 15 min (Fig. 4b). We have at present no explanation for the more pronounced action of chloroquine on the ATP content of cells incubated without glucose compared to cells incubated with glucose, especially because we observed an about 2 times higher intracellular accumulation of chloroquine in the presence of glucose at pH 7.5 (Table 3). A further argument for metabolic effects of chloroquine not being related to inhibition of proteolysis resulted from the measurement of the rates of fermentation of glucose and of accumulation of ethanol under aerobic conditions. Chloroquine, at 10 mM inhibited glucose consumption to about 50% and completely prevented the aerobic formation of ethanol (Fig. 5).

From ^{31}P NMR studies with *Saccharomyces cerevisiae* (Navon et al. 1979), the average pH values of vacuoles and cytosol were estimated to be 6.5 and 7.2, respectively. In addition to these ^{31}P NMR investigations, our findings offer microscopic evidence for a more acidic pH of the yeast vacuoles compared to the cytosol. Since unphysiological substrates are used for routine assays of vacuolar and other proteinases and since the pH optimum of proteolytic activities depends on the structure and conformation of the substrate as exemplified with yeast proteinase A (Meussdoerffer et al. 1980), it can at present not be decided how far overall inhibition of vacuolar proteolytic acitivities by chloroquine is caused by an increase of the pH value or by direct interaction of chloroquine with proteinases.

Acknowledgements. We thank Dr. F. Meussdoerffer for supplying purified proteinase A, Dr. H. Matern for the gift of purified proteinase B and carboxypeptidase Y, Prof. Wiemken (ETH Zürich) for the estimation of the fluorescence microscope photographs, and Prof. Y. Natori and Dr. I. Beck for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 206 and the Verband der Chemischen Industrie e. V. (Fonds der Chemischen Industrie). The excellent assistance of Mrs. L. Leuschel during the preparation of this manuscript is gratefully acknowledged.

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Received July 6, 1983/Accepted October 5, 1983