ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 3/2017
503–506
https://doi.org/10.18388/abp.2017_1510

Regular paper

Developmental changes in the levels and redox potentials of main hemolymph thiols/disulfides in the Jamaican field cricket *Gryllus assimilis*

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Main thiols and disulfides were determined in the hemolymph of the Jamaican field cricket *Gryllus assimilis* at various developmental stages. On the basis of these data, redox potentials of the glutathione, cysteine and homocysteine redox systems were calculated. The concentrations of all thiols studied decreased during development (at a stage of 6 molts) with respect to young crickets, and increased again in adult insects. Redox potentials of the glutathione and cysteine systems increased from values of –131.0±5.6 mV and –86.9±17.1 mV, respectively in young crickets to –58.0±3.6 mV and –36.1±4.2 mV, respectively, at the stage of 6 molts and decreased to values of –110.4±24.8 mV and –66.3±12.2 mV, respectively, in adult insects. Redox potentials of the glutathione and cysteine systems in the hemolymph of young and adult insects were similar to those reported for human plasma.

Key words: insect, *Gryllus assimilis*, hemolymph, glutathione, cysteine, homocysteine, redox potential, redox environment

Received: 22 January, 2017; revised: 03 May, 2017; accepted: 08 May, 2017; available on-line: 27July, 2017

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Abbreviations: CMQT, chloro-1-methylquinolinium tetrafluoroborate; CSH, cysteine; CSSC, cysteine; GSH, glutathione; GSSG, oxidized glutathione; HCSH, homocysteine; HCSSHC, homocystine; PCA, perchloric acid; TCA, trichloroacetic acid; TCEP, tris-(2-carboxyethyl)phosphine

INTRODUCTION

Comparative biochemistry has accumulated a wealth of data demonstrating both the biochemical unity and diversity of the living world. Basal amino acids, including cysteine, occur ubiquitously in living cells although some groups of organisms contain atypical amino acids or their analogs. Similarly, the tripeptide glutathione is almost universally present in living organisms although some analogs of this molecule are present e.g. in some bacteria (Newton & Fahey, 2002), trypanosoma (Manta *et al*., 2013), and in some plant families (Zi *et al*., 2010).

Glutathione, as a substrate of glutathione peroxidase, is one of the main factors responsible for the removal of peroxides, first of all hydrogen peroxide and, as a substrate of glutathione *S*-transferase, is one of the principal compounds involved in phase II detoxification of exogenous and endogenous electrophiles (Meister & Anderson, 1983; Forman *et al*., 2009). Moreover, glutathione is the most significant intracellular redox buffer, the system of reduced glutathione (GSH)/oxidized glutathione (GSSG) being the main determinant of cellular "redox environment" and an important contributor to the extracellular "redox environment"(Schafer & Buettner, 2001). In mammalian blood, GSH is mainly present in blood morphotic elements, its concentration in erythrocytes being about 2 mM and that in blood plasma being about 2 orders of magnitudes lower.

The concentration of glutathione and the ratio of reduced to oxidized glutathione concentration decrease during aging of Drosophila (Rebrin *et al*., 2004). It was of interest to check whether analogous changes take place in the insect hemolymph during development and aging of another insect species (more appropriate to study hemolymph due to bigger size). The aim of this study was thus to analyze the concentrations and redox potentials of three main thiol/disulfide redox systems during development of the Jamaican field cricket *Gryllus assimilis*.

MATERIALS AND METHODS

Instrumentation. The analyses were performed on a 1220 Infinity LC system from Agilent equipped with binary pump integrated with two-channel degasser, autosampler, column oven and DAD detector. A 5 µl injection introduced the samples with the aid of an autosampler and chromatographic separation was achieved on a Zorbax SB C-18 (150×4.6 mm) column (Agilent Technologies, Waldbronn, Germany), packed with 5 µm particles. For instrument control, data acquisition and analysis, OpenLAB CDS ChemStation Edition was used. For sample homogenization an IKA T10 basic homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) was used.

Insects. Jamaican field cricket *Gryllus assimilis* was purchased from a local animal store. In the middle of the intermolt period hemolymph was withdrawn from cold-immobilized insects with a syringe and mixed with an anticoagulant (62 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid) in a ratio of 4:1. The anticoagulant contained CMQT when reduced thiols were estimated. Hemolymph samples from several insects (2–9, depending on the size) of the same age group were combined for analysis.

Chemicals and reagents. All chemicals used throughout this study were of analytical reagent grade except of 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), which was synthesized as described previously (Bald & Glowacki, 2001). Reduced thiols: cysteine (CSH), glutathione (GSH) homocysteine (HCSH) and their oxidized forms were received from Sigma (St. Luis, MO, USA). Hydrochloric acid (HCl), perchloric acid (PCA), sodium hydroxide (NaOH), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \times 7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \times 2H_2O$) were from J.T. Baker (Deventer, The Netherlands). Trichloroacetic acid (TCA) and tris-(2-carboxyethyl)phosphine (TCEP) were from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was from J.T. Baker (Deventer, The Netherlands).

Stock solutions. Stock solutions of 0.01 M CSH, GSH, HCSH and their symmetrical disulfides needed as standards were prepared by dissolving appropriate amount of the compound in 2 ml of 0.1 M hydrochloric acid and diluting to the volume of 10 ml. These solutions were kept at 4°C for several days without noticeable change of the thiol content. The working solutions were prepared, by appropriate dilutions with water as needed, and processed without delay.

Stock solutions of 0.25 M TCEP and 0.1 M CMQT were prepared by dissolving appropriate amount of the compound in 1 ml of 0.2 M phosphate buffer (pH 7.4) before analysis.

Chromatography. Quantification of thiols was carried out by a modification of our previously described procedure (Glowacki & Bald, 2009). A gradient elution was used with a flow rate of 1 $ml \times min^{-1}$. The elution profile was as follows: 0–8 min, 11–40% B; 8–12 min, 40–11% B; (A/B, v/v). Elution solvent B was acetonitrile and solvent A was 0.1 M TCA water solution, adjusted to pH 1.7 with 1 M NaOH. The detection and quantification were by UV absorbance at 355 nm. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at real time of analysis, with a corresponding set of data obtained for authentic compounds.

Analytical procedures. Determination of total thiols. Sixteen µl of the hemolymph mixed with 4 µl of the anticoagulant was supplemented with 20 µl of 0.1 M phosphate buffer (pH 7.4) were added followed by 2 µl of TCEP stock solution. The mixture was vortexed and put aside for 15 min. Then 2 µl of stock solution of CMQT was added. After 4 min the reaction mixture was acidified with 10 µl of 3 M PCA followed by centrifugation (12000×*g*, 10 min). Ten µl of the supernatant was then transferred into the HPLC system.

Determination of reduced thiols. Thirty five µl of the hemolymph was mixed with 10 µl of the anticoagulant and 5 µl of the stock solution of CMQT. After 2 min the reaction mixture was acidified with 10 µl of 3 M PCA. Precipitated proteins were separated by centrifugation during 5 min at 12000×*g* and 10 µl of the resulting supernatant was injected onto the HPLC column.

Statistics. All measurements were done with 3-5 independent samples. Statistical significance of differences calculated using the Mann-Whitney "U" test.

RESULTS AND DISCUSSION

We analyzed the main thiols and disulfides in the hemolymph from different age groups of *Gryllus assimilis* using HPLC methods. These methods are capable of evaluating thiols, among them CSH, GSH and HCSH, as well as disulfides including cystine (CSSC), glutathione disulfide (GSSG) and homocystine (HCSSHC) (Glowacki *et al*., 2012; Kusmierek *et al*., 2011). The use of CMQT prevents thiol oxidation during sampling, which is a common source of artifacts in thiol analysis. Recently, a more sensitive method has been proposed for determination of cysteine and glutathione in insect hemolymph, allowing for assays in hemolymph samples from a single *Drosophila* imago (Borra *et al*., 2015). However, it does not allow to discriminate between reduced and oxidized forms of the thiols, being thus inappropriate for studies of the redox status of the hemolymph.

We determined main thiol compounds in the hemolymph of *G. assimilis* at various stages of development: in a group of 4th instar nymphs (after 3 molts, designated as young, Y), in a group of 7th instar nymphs (after 6 molts, M) and in adult crickets (A). The three main thiols present in the hemolymph were: glutathione>cysteine>>homocysteine. The concentrations of all thiols showed a characteristic change during the development, being the highest in young crickets (4th instar), then decreasing during maturation and being restored to higher levels in adult insects (the increase in adult crickets being statistically significant for GSH) but not for HCSH (Table 1).

Redox potentials calculated on the basis of thiol and disulfide concentrations using the Nernst equation showed the lowest redox potential for the glutathione system and the following sequence of the redox potentials for the other systems: glutathione system<cysteine system<homocysteine system. The developmental changes in the concentrations of thiols and disulfides resulted in changes of the thiol/disulfides ratios and of the redox potentials of the respective redox systems: increase in the middle stage of development with respect to the young stage of development and a decrease again in the adult stage (the latter being statistically significant for GSH). The redox potential of the homocysteine system did not decrease in the adult stage but the values for this

Table 1. Concentrations of main thiols and disulfides in the hemolymph at various developmental stages of *Gryllus assimilis*. Values are expressed in µM, means of 5 experiments ± S.D.

Developmental stage[$]	GSH	CSH	HCSH	GSSG	CSSC	HCSSHC
Y	59.2±10.8 *M	19.6±12.3 *M	1.47±0.71 *M	107.6±17.7	62.9±16.1	3.19±1.01
M	6.7±2.1 *Y *A	4.8±1.9 *Y	0.32±0.25 *Y	71.4±11.9	48.2±11.7	1.72±0.95
A	37.1±15.4 *M	7.5±2.5	0.55±0.08	90.9±48.5	50.4±21.0	1.49±0.92

[$]Y, 4th instar; M, 7th instar; A, adult insects. tatistical significance of differences: *$P<0.05$ with respect to Y, M or A, respectively.

Table 2. Thiol/disulfide concentration ratios and redox potentials of the glutathione, cysteine and homocysteine systems in the hemolymph of various developmental stages of *Gryllus assimilis* (mean ± S.D.).

	Concentration ratio			Redox potential [mV]		
Crickets	GSH/GSSG	CSH/CSSC	HCSH/HCSSHC	Glutathione system	Cysteine system	Homocysteine system
Y	0.56±0.14 *M	0.44±0.16 *M	0.35±0.22 *M	–131.0±5.6 *M *A	–86.9±17.1 *M *A	–59.2±10.3 *M
M	0.10±0.04 *Y *A	0.18±0.08 *Y	0.10±0.02 *Y	–58.0 ±3.6 *Y *A	–36.1±4.2 *Y	–43.8±6.9 *Y
A	0.47±0.24 *M	0.33±0.11	0.18±0.12	–110.4±24.8 *Y *M	–66.3±12.2 *Y	–43.9±3.5

[§]Y, 4th instar; M, 7th instar; A, adult insects. Redox potentials were calculated taking into account that redox potentials of thiol/disulfide couples are lower by 0.024 mV at pH 7.4 than at pH 7.0, so that standard potentials of glutathione, cysteine and homocysteine at pH 7.4 are –0.264 V, –0.244 mV and –0.244 mV (Jocelyn, 1967; Schafer & Buettner, 2001). Statistical significance of differences: *$P<0.05$ with respect to Y, M or A, respectively.

system may be less reliable due to the low concentration of homocysteine (Table 2).

The redox environment calculated as the sum of the products E_i x [reduced species]$_i$, where E_i is the half-cell reduction potential for a given redox system and [reduced species]$_i$ is the concentration of the reduced species of that redox system (Schafer & Buettner, 2001), amounted to –9.5 mM mV for young (4th instar) cricket nymphs, –0.6 mM mV for 7th instar nymphs and –4.6 mM mV for adult crickets when glutathione, cysteine and homocysteine were taken into account.

The redox systems of glutathione and cysteine have been extensively studied in human blood plasma. Interestingly, the redox potentials of the glutathione and cysteine systems of human blood plasma (–137±9 mV and –80±9 mV, respectively) were similar to those found in the hemolymph of young and adult crickets in this study.

A significant correlation between the redox potentials of the glutathione and cysteine systems was observed in human blood plasma (Jones *et al*., 2000), resembling those during the development of the cricket observed in the present study. In human blood plasma, the redox potential of glutathione increases with age and in diseases, reflecting progressive oxidation of the extracellular milieu (Samiec *et al*., 1998; Johnson *et al*., 2008). Similar age-related redox changes in blood plasma glutathione and cysteine systems were also found in chimpanzee *Pan troglodytes,* rhesus monkey *Macaca mulatta* (Paredes *et al*., 2014) and marmoset *Callithrix jacchus* (Roede *et al*., 2013). Redox potentials of both glutathione and cysteine systems reflect systemic oxidative stress as exemplified by increased values of these potentials in smokers *vs* non-smokers (–128±18 mV *vs* –137±17 mV and –64±16 mV *vs* –76±11 mV, respectively) (Moriarty *et al*., 2003). The redox potentials of the glutathione and cysteine systems in human blood plasma were found to be subject to diurnal variation (Blanco *et al*., 2007).

While the existence of similar relationships has to be examined in the insect hemolymph, the present study revealed an increase in the oxidation state of all main hemolymph redox systems in the final stage of the development of *Gryllus assimilis*. The reasons for this phenomenon are not clear but may be related to reaching maturity and represent an initial state of progressive oxidation of hemolymph redox systems during aging as postulated by the redox stress theory of aging (Sohal and Orr, 2012). In agreement with this theory, the GSH/GSSG ratio increased in homogenates of *Drosophila melanogaster* during aging of the insects (Rebrin *et al*., 2004). Intracellular glutathione had a dominant influence on these results but homologous changes can be expected also to occur in the insect hemolymph.

Acknowledgement

This study was supported by the University of Rzeszow and the University of Lodz.

We are indebted to Dr. hab. Roma Durak for providing the material and help in the hemolymph collection.

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