Species-specific characterisation of thiolate - disulfide transitions; the influence of molecular structure on disulfide bridge formation and kinetics

PhD thesis

Zoltán Faragó PharmD.

Doctoral School of Pharmaceutical Sciences

Semmelweis University

Supervisor: Béla Noszál PharmD, DSc

Advisor: Arash Mirzahosseini PharmD, PhD

Official reviewers: Gergely Agócs PharmD, PhD

Tamás Jakusch, PhD

Head of the Complex Examination Committee:

Romána Zelkó PharmD, DSc

Members of the Complex Examination Committee:

Pál Perjési PharmD, CSc

Krisztina Ludányi, PhD

Budapest

2022

Introduction

As a part of cellular metabolism, reactive oxygen species (ROS) are normally produced by biological systems during cellular respiration. At low or moderate levels, ROS exert their beneficial effects on redox homeostasis. These species play a key role in cell proliferation signalling, enhance immunologic defence, support physiological functions, lower the risk of various diseases, including cancer. Excessive levels of ROS shift the balance between oxidants and antioxidants in favour of oxidants, generating oxidative stress and causing oxidative damage in cells. Increased level of oxidative stress takes major part in formation and progression of cancer, Alzheimer's disease, sporadic amyotrophic lateral sclerosis, Parkinson's disease. Thiol functional group containing molecules are the main protectors against ROS in living organisms. The thiol group is found in every cysteine residue, however not every cysteine-containing peptide is capable to neutralize ROS. Glutathione (GSH) is one of the well-known members of easily oxidizable cysteine containing endogenous antioxidants. GSH depletion and GSH-related enzyme deficit are involved in the pathology of several neuropsychiatric and

neurodegenerative diseases, such as autism, schizophrenia, bipolar disorder, Parkinson's, and Alzheimer's disease. Moreover, the redox imbalance of glutathione may be a primary cause of these disorders. Besides this protective function against ROS, disulfide bonds are important factors to provide a tertiary structure for proteins. Disulfide bonds are found in nearly one third of the proteins in a eukaryotic cell. Proper disulfide bonds ensure stability to a protein, decreasing the number of further entropic choices of the folding progression toward the native state.

Conotoxins, a group of neurotoxic peptides, isolated from the venom of the marine cone snail (genus *Conus*), have been chosen in this work to examine the disulfide forming patterns of peptides. The main reason we selected this class of peptides is their relatively small size; they consist only of 10 - 60 amino acid residues, making them one of the smallest peptides in nature that contain multiple disulfide bonds. We used in our experiments a reduced derivative of α-conotoxin MI with the sequence of GRCCHPACGKNYSC (see Figure 1.).

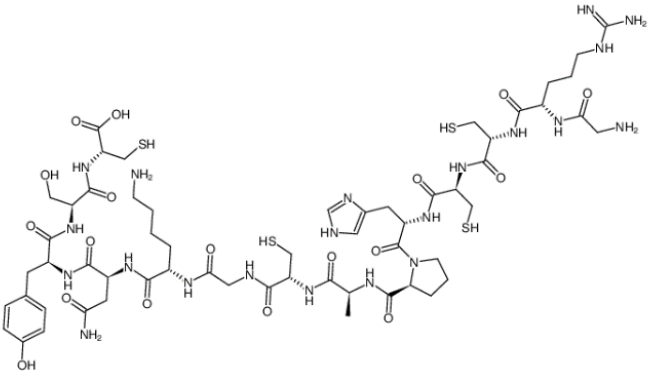


Figure 1. Primary structure of the α-conotoxin's derivative MI (with the sequence of GRCCHPACGKNYSC)

Objectives

Our main objectives were to investigate what kind of features and reaction conditions influence the rate and quality of the disulfide bridge formation.

Our first aim was the investigation of the pH – reaction velocity relation, secondarily the definition of species – specific kinetic constants (which are pH-independent parameters) for the thiolate – disulfide transition reactions, using the simplest model compounds, mercaptoethanol (MSH) – cystamine (CSSC), mercapthoethanol-disulfide (MSSM) and cysteamine (CSH). With these models we were able to ascertain the through-bond influence of a two-

carbon-distance protonated amino and hydroxyl group for the thiol-oxidation, which can be used for an approximation of glutathione's redox attribution.

After the characterisation of the reduced α-conotoxin-MI NMR spectra, our second aim was to determine the species- and site-specific constants, including 4 thiolate pK_a values. With these p*K*a values our purpose was to find out how p*K*a values affect the disulfide bond formation. Based on our hypothesis, the thiolate groups with the highest protonation constants and therefore highest oxidation propensities will form disulfide bonds first, unless a strong alternate conformational preference exists.

Methods

Thiol-disulfide reactions

For the characterization of thiolate – disulfide reactions between MSH – CSSC and MSSM – CSH we used quantitative ^{1}H NMR. The solvent in every case was an aqueous solution with H_2O: D_2O, 95: 5 V/V%, with 0.15 mol / dm^3 ionic strength. NMR spectra were recorded on a Varian 600 MHz spectrometer at 298 K. pH values were determined by internal indicator molecules, optimized for NMR.

The exact concentrations of the reagents before the reaction, were determined by high precision gravimetric analysis. D_2O, acetone (as chemical shift reference), and a pH indicator (sodium acetate or imidazole), which also served as a concentration standard, were added to the solutions. The pH of the solutions was adjusted with aqueous phthalate buffer (0.1 mol / dm^3) and hydrochloric acid or sodium hydroxide. The concentration of the reagents and products as a function of time, can be calculated from NMR spectra after the assignment (see Figure 2. for details), and integration.

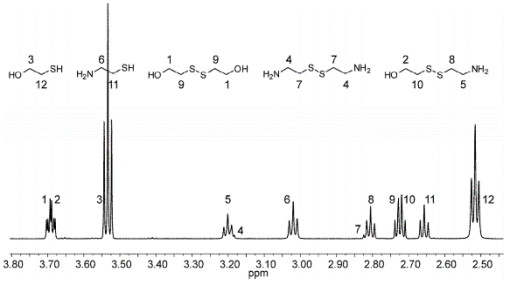


Figure 2. 1H NMR spectrum of the 12 different methylene peaks of the five different compounds (from left to right: MSH,

CSH, MSSM, CSSC and MSSC) on pH = 4.8 (Mirzahosseini A. et al, 2018).

We have got 10 sets of measurements for the MSH + CSSC reaction, and 7 sets of measurements for the MSSM + CSH reaction on different pH-s. One set of measurements included 15 – 30 spectra, resulting in 75 – 150 concentration data on one pH, as a function of time.

ACD/NMR Processor Academic Edition v12.01 software package and Origin Pro 8 have been used for NMR spectra procession and data analysis. All the possible reactions between the microspecies and their rate constants are depicted in Figure 3.

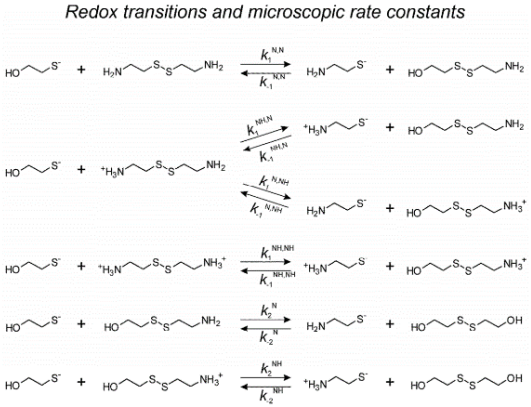


Figure 3. Summary scheme of redox transitions and pH – independent microscopic rate constants. The details of the

notation are in chapter Discussion (Mirzahosseini A. et al, 2018).

Conotoxin

pH-titration

The peptide was titrated in D_2O solution at 298 K and 0.15 mol / dm^3 ionic strength, the pH was adjusted with DCl and NaOD. The methyl signal of internal DSS was used as a chemical shift reference. A D_2O solution was used to perform the titration since the methylene ^1H signals that could be used for the evaluation are close to the water signal and are often suppressed in ^1H measurements with solvent suppression.

Structure

A 0.5 mmol / dm^3 aqueous solution (containing 5 V/V% D_2O) of the peptide was prepared at pH = 4.5 (298 K and 0.15 mol / dm^3 ionic strength), for the 3D structure determination. pH = 4.5 was chosen because the average relative charge – pH curve has a plateau at this point. The peptide occurs predominantly in a +3 charged state at pH = 4.5. The entire assignment of the peptide was performed using NOESY and COSY spectra and confirmed with TOCSY spectrum. The chemical shift reference was the methyl signal of the internal DSS in the solution. After the

titration of the peptide the circular dichroism spectrum of the peptide was recorded at selected pH values to cover the entire pH region of the protonation. 2D NOESY spectra were studied for structure determination, at pH = 4.5 medium, with CcpNmr Analysis V2 and ARIA. The structure refinement created 7 alternative structures.

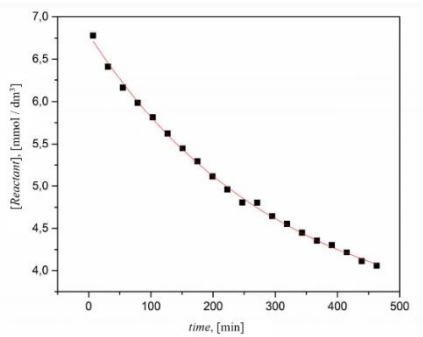
Results

Thiol-disulfide reactions

Applying the results of the 17 sets of measurements, regression analysis was performed for both directions (forward and reverse), for each reactant in every set, using the equation of exponential decay:

(1) $[reactant] = A * e^{\frac{-t}{\tau}} + B$

Where A is the initial concentration of the reactant, e is Euler's number, τ is the exponential time constant, and B is the correction factor. The time constant τ is the amount of time, that an



exponentially decaying quantity (i.e., concentration) takes to decay by a factor of $1/e$. $1/e$ is approximately 0.368, so τ is the amount of time that the concentration takes to decay to 36.8% of its original amount. Correction factor B represents the concentration of the reactant after the reaction, in the equilibrium. For the goodness-of-fit see Figure 4.

Figure 4. The fitted exponential decay of mercaptoethanol reactant at pH = 4.62 (Mirzahosseini A. et al, 2018).

To calculate the apparent rate constant, the following transformations were performed: only the first step of the reaction affects the consumption of the reactants at t = 0 s. These can be formulated for forward and reverse directions as follows:

(2) $v_1 = k_1[MSH]_0[CSSC]_0 = \frac{-d[MSH]}{dt} = \frac{-d[CSSC]}{dt}$

(3) $v_{-2} = k_{-2}[MSSM]_0[CSH]_0 = \frac{-d[MSSM]}{dt} = \frac{-d[CSH]}{dt}$

Where the subscript zero after the square bracket represents initial concentration. The derivative terms of equation (2) and (3) were acquired from the derivative form of equation (1). With this method apparent rate constants can be calculated for both directions, as a function of pH. The microscopic rate constants were determined from rate equations, composed of microspecies concentrations, and apparent rate constants. The microscopic $k_{-1}^{NH,NH}$ and k_2^{NH} rate constants were calculated using the microscopic, species–specific redox equilibrium constants. The microscopic redox equilibrium constants are defined in equations (4) and (5):

(4) $K_1^{REDOX,a-l} = \frac{k_1^{NH,NH}}{k_{-1}^{NH,NH}} = \frac{[g][e]}{[a][l]}$

(5) $K_2^{REDOX,a-e} = \frac{k_2^{NH}}{k_{-2}^{NH}} = \frac{[g][c]}{[a][e]}$

The conditional equilibrium constants were determined at different pH values approaching from both directions, resulting:

(6) $\log (K_1^{REDOX,a-l}) = 1{,}91 \pm 0{,}07$

(7) $\log (K_2^{REDOX,a-e}) = 0{,}77 \pm 0{,}09$

For calculated macroscopic and microscopic rate constants see Table 1.

Forward direction			Reverse direction		
pH	$\log k_1$	$\log k_1^{NH,NH}$	pH	$\log k_{-2}$	$\log k_{-2}^{NH}$
4.20	-2.385	2.553	4.30	-3.097	0.573
4.35	-1.990	3.300	4.42	-3.503	0.811
4.37	-1.940	3.330	4.52	-3.135	1.200
4.40	-2.260	2.990	4.82	-2.650	1.059
4.41	-2.109	3.122	5.04	-2.488	0.964
4.62	-1.702	3.315	5.05	-2.367	0.540
4.92	-1.382	3.339	5.24	-2.395	0.740
5.08	-1.385	3.175			
5.31	-1.096	3.234			
5.66	-0.976	3.009			

Table 1. The macroscopic / apparent and microscopic rate constants are presented in log units. The dimension of the rate constants is $mol^{-1}*dm^3*s^{-1}$ (Mirzahosseini A. et al, 2018)

The consequently determined microscopic rate constants are depicted in Figure 5.

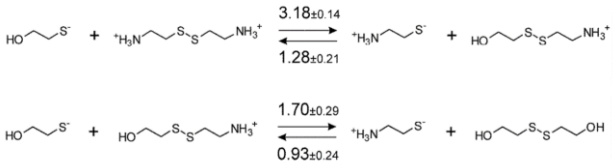


Figure 5. The determined $k_1^{NH,NH}$, $k_{-1}^{NH,NH}$, k_2^{NH} and k_{-2}^{NH} microscopic rate constants in log units with the corresponding arrows. The dimension of the rate constants is $mol^{-1}*dm^3*s^{-1}$. (Mirzahosseini A. et al, 2018)

Conotoxin

From the NMR – pH titration the pKa values of the α-Conotoxin MI derivative have been determined. When βH signals were not observable, αH signals were used as alternatives. (See Figure 6. for details)

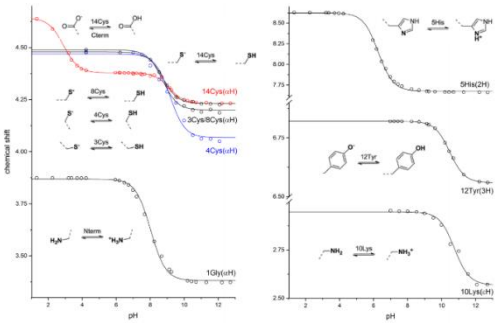


Figure 6. The 1H NMR titration data and fitted curves of the peptide. (Faragó Z. et al, 2021)

pH = 4.5 was chosen for the 3D structure determination because the average relative charge – pH curve has a plateau at this point. We also attempted to determine the structure of the peptide at a high pH value, where every protonating group is deprotonated (except for the arginine guanidinium), resulting in an average charge of -5. Molecular dynamics simulations were used for the conformation search of the peptide (with +3 and -5 charges).

Conclusions

Thiol – disulphide reactions

Thiol oxidation was examined on CSSC – MSH and MSSM – CSH systems to model the redox properties of glutathione with relatively simple and easier to operate molecules. Measuring the concentrations of the reactants and products with quantitative ^{1}H-NMR and using a new method for data processing, we have determined pH-dependent apparent rate constants between 4 < pH < 6. Based on the calculated relative abundance of microspecies and equilibrium constants, pH independent microscopic rate constants have been defined for $k_1^{NH,NH}$, $k_{-1}^{NH,NH}$, k_2^{NH} and k_{-2}^{NH}. The curve of the apparent rate constants as a function of pH shows an exponential – like rise under acidic conditions (4 < pH < 6) although it is expected to be a sigmoidal under basic conditions. The microscopic rate constants were significantly different from each other.

Further measurements under basic conditions could be useful in the future to quantify additional microscopic rate constants and to observe the sigmoid curve of the apparent rate constant – pH function. However, it is necessary to

use other technics (for instance stopped flow method) for these goals, because the rate of the reactions gets too fast for NMR measurements.

Conotoxin

In other experiments we studied how physico – chemical properties of the thiol groups affect the disulfide bond formations. For the investigation the reduced derivative of α-conotoxin MI was chosen. After ^{1}H-NMR, ^{1}H – ^{13}C HSQC and COSY measurements the assignment of the ^{1}H signals were complete. The pK_a values were determined. Based on previous work, we could calculate the group – specific standard redox potentials for the four cysteine residues. These redox potential values revealed that the thiol groups with the highest oxidation propensities will form disulfide linkage first, if the thiols are close enough to each other. CD measurements and molecular dynamics simulations verified the lack of any classical secondary structure of the peptide at pH = 4.5. As a result, we can state that the primary structure of the thiols and disulfides influences the physico – chemical properties, for instance the electron density around the sulfur atoms and the pK_a values, due to inductive effects. The reactivity of these molecules shows clearly different apparent and

microscopic rate constants. Also, disulfide bonds tend to form first between cysteine residues of high protonation constants and low redox potentials.

Bibliography of the candidate's publications

Faragó Z, Mirzahosseini A, Horváth D, Pálla T, Horváth P, Perczel A, Noszál B. (2021) Solution Structure and Acid-Base Properties of Reduced α-Conotoxin MI. Chem Biodivers, 18, e2100464

Mirzahosseini A, **Faragó Z**, Noszál B. (2018) Determination of pH-independent rate constants of thiolate-disulfide redox transitions. New J Chem, 42: 11653-11659.