The paradoxical influence of the pKa on the reactivity of thiols and its biological relevance

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Abstract

Background. Not much is known about the factors that influence thiol reactivity. In this paper we will investigate the paradoxical influence of the acid dissociation constant (pKa). The pKa influences the deprotonation of a compound. This deprotonation gives thiols a negative charge and therefore increases reactivity. Compounds with a low pKa have a higher percentage of deprotonation, this will lead to a higher reactivity. On the other side, thiols with a higher pKa have a higher electron density, and are therefore also more reactive. We want to recognize this paradox and investigate what the exact contribution of these two paradoxical mechanisms is with respect to the reactivity of thiols. Furthermore we will discuss the biological importance of this study with respect to the anti-oxidant system and drug conjugation.

Methods. The reactivity of the thiols was determined by calculating the rate constant of these compounds. This was done for different thiols in reaction with 1-chloro-2,4-dinitrobenzene. To do this we created a pseudo first order reaction by adding an excess of CDNB. The thiols we used in this reaction were glutathione, 5-thio-2-nitrobenzoic acid, 2-mercaptoethanol and 2-mercaptobenzothiazole. The reaction rates were measured, using a spectrophotometer and eventually the reaction constant was calculated and transformed into the reactivity of the thiolate ion.

Results. The reaction constants from the different thiols are dependent on the fraction thiolate ion. This was converted to the reactivity of the thiolate ion. The reactivity of thiolate ion decreases as pKa value increases.

Conclusion. There is indeed a paradoxical influence of the pKa value on the reactivity of thiols. Increases in pKa lead to decreases in reactivity of the thiolate ion but to increases in the fraction of the thiolate ion. Eventually there was no mechanism that had the biggest contribution to the reactivity for all conditions. Apparently this is dependent on the pH.

We advice drug researchers to keep this paradox in mind in order to estimate the reactivity of thiols.

Keywords

Thiol reactivity, pKa, Glutathione, 2-mercaptoethanol, 5-thio-2-nitrobenzoic acid

Introduction

The structure of thiols

Thiols are molecules with a sulfhydryl group (-SH). The bond between sulfur and hydrogen is a nonpolar covalent bond, due to their small difference in electronegativity. This makes it relatively easy for the sulfur to lose the proton (H^+). Thiols are therefore mostly weak acids. The reactivity of thiols is caused by the sulfur atom, which possesses two non-bonding electron pairs. This makes a thiol a weak nucleophile. The strength of the thiol as a nucleophile greatly enhances when the thiolgroup loses its hydrogen proton, thereby becoming a so-called thiolate ion. This deprotonation leaves the thiol with a negative charge, thereby making the thiol much more reactive towards electrophiles [1]. The degree of deprotonation of the thiol is dependent on the pH of the environment and different for every thiol, determined by the acid dissociation constant (pKa) of the thiol-group. The deprotonation occurs according to the following reaction:

$$RSH + H_2O \leftrightharpoons RS^- + H_3O^+$$

This pKa-value shows towards which side this equilibrium is shifted and is therefore a measure for the strength of an acid. This is different for every compound. This equilibrium can be calculated using the Henderson-Hasselbalch equation:

This equation shows that the equilibrium in acids with a high pKa will be shifted towards the protonated RSH state, where acids with a low pKa will be mostly in their deprotonated RS^- state. This is determined by the pH, so if pH >> pKa + 2, almost 100% of the thiol will be deprotonated [2]**.**

The paradoxical reactivity of thiols

The interesting thing about the influence of pKa on thiol-reactivity is that it is somewhat paradoxical. A low pKa-value will make it very favorable for the compound to lose its proton, thereby increasing the amount of deprotonation and thereby the reactivity of the

thiol. Whereas a high pKa will cause the compound to be mainly in its R-SH state, thereby reducing the reactivity of the thiol. On the other side, the pKa-value of a compound also influences the intrinsic reactivity of the thiol. Compounds with a high pKa value have an increased electron density on the sulfur-atom. This causes the strong binding between sulfur and hydrogen, which explains why their equilibrium is shifted towards the R-SH state. Vice versa for compounds with a low pKa, which have a low electron density on the sulfur atom, the hydrogen proton is lost quite readily. The electron density influences the intrinsic reactivity of a compound, resulting in a higher intrinsic reactivity for compounds with a high pKa and a lower intrinsic reactivity for compounds with a low pKa. The effects of the electron density and protonation with respect to the pKa value are therefore paradoxical.

The aim of this study

In this study we want to investigate what the exact influence of the pKa is on the reactivity of the thiols. We expect to recognize this paradox in the reactivity of the different thiols. Furthermore we want to investigate what the exact contribution is of the deprotonation and the electron density in order to find out which one of these two has the biggest influence on the reactivity of the thiols.

Biological relevance

The biological relevance of this study lies in the importance of thiols. Thiols are bioactive substances that play essential roles in the cellular biochemistry of all organisms. Important functions include roles in protein folding, regulation of oxidative stress and drug detoxification. In particular the amino acid cysteine plays a central role in these functions. It supports the tertiary structure of proteins by oxidizing into disulfide bridges, thereby forming cystine units. But cysteine can also form the active region of certain proteins, caused by its nucleophillic properties. This is the case in the well-studied tripeptide glutathione. Glutathione consists of three amino-acids; glutamic acid, cysteine and glycine and is abundantly present in our cells (millimolar range) which also suggests its importance. It is involved in drug conjugation and plays a very important role in the reduction of hydrogen peroxide and tocopherol radicals, thereby reducing oxidative stress and contributing to the anti-oxidant network [3, 4]. However we will not be focusing on these reduction reactions, but rather on nucleophillic reactions in which thiols are involved. These nucleophillic reactions are pivotal in drug detoxification. Most drugs in the human body undergo phase 1 and phase 2 reactions in order to be excreted. In phase 1 they are converted into more polar substances by adding or unmasking reactive –OH, -NH_2, -O- or –

SH groups, this is done by oxidation, reduction or hydrolysis reactions. This often results in an inactive metabolite, however in some specific cases this reaction product is the active drug [5]. If these phase 1 products are polar enough, they can immediately be excreted. In most cases however, these products need to be converted into a highly polar conjugate, this is done by phase 2 reactions. Phase 2 reactions conjugate the reactive groups formed in phase 1. Reaction products from phase 1 reactions can be either electrophilic or nucleophillic. The nucleophillic compounds can be conjugated by different enzymes, but the electrophillic compounds are frequently conjugated to glutathione via the enzyme Glutathione-S-transferase. This enzyme couples the glutathione thiolate ion to the drug, thereby creating a much more polar conjugate that can easily be excreted [6, 7].

Another intriguing example of a protein that contains cysteine residues is Keap1, which is important in the defense against oxidative stress. Modification of Keap1 induces an anti-oxidant response that can counteract oxidative stress. Oxidative stress is the accumulation of certain harmful compounds called reactive oxygen species (ROS) in the cell, due to the disbalance between ROS formation and antioxidant capacity. These molecules damage the membrane, DNA and proteins inside the cell. Thereby leading to harmful effect such as mutations, which can increase the risk of multiple diseases including different types of cancer [8]. These molecules can arise internally, for instance the formation of superoxide radical by mitochondria out of oxygen[9], or the formation of superoxide radical by phagocytes in order to kill microbes [8]. ROS are therefore a side effect of biologically required processes, and formation cannot be prevented. As a response, the body has developed an impressive antioxidant-system, which tries to counteract the reactivity of these ROS and prevent them from accumulating. The transcription of many antioxidant genes, together with drug conjugation-related phase 2 genes is regulated by Nrf2. Nrf2 is a transcription factor that induces the transcription of these genes by heterodimerizing with Maf-proteins and subsequently binding to the "antioxidant responsive element" (ARE) on the DNA [10-13]. Target genes of Nrf2 are involved in GSH biosynthesis [10, 13], reactive oxygen scavenging [10, 13, 14], drug metabolism [10, 11, 13] drug transport [10, 11, 13] and detoxification of heavy metals [10]. This process is negatively regulated by the protein Keap1. Under normal conditions, Keap-1 inhibits Nrf2 in two ways. Keap-1 is bound to actin filaments, binding of Keap1 to Nrf2 therefore prevents Nrf2 from translocating to the nucleus. Besides, Keap-1 can also bind to Cul3, a component of the E3-ligase complex. This binding causes Nrf2 to be ubiquitinated and subsequently degraded by a proteasome [10, 15]. Keap1 contains 25 conserved cysteine residues [11], indicating the importance of these groups. The reactive thiols of these cysteines can be modified by electrophiles, thereby disturbing the Keap1-Nrf2 complex and allowing Nrf2 to translocate to the nucleus and

activate the transcription of the different target-genes [11, 15]. It can therefore be said that Keap-1 sensors oxidative and xenobiotic stressors and reacts by initiating an antioxidative response (figure 1).

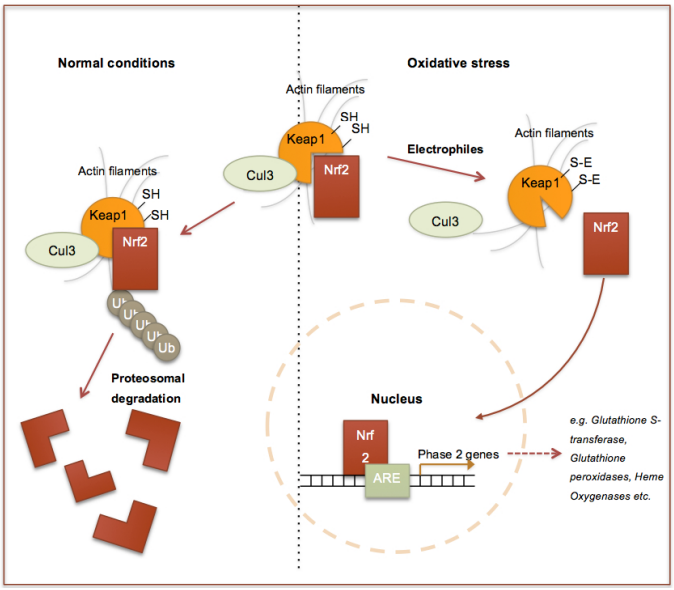

Figure 1. The Keap1-Nrf2 pathway. On the left side, the pathway under normal conditions is shown. In this case Keap1 is bound to Nrf2 and Cul3. Cul3 ubiquinates Nrf2 and Nrf2 is subsequently degraded. On the right side the pathway is shown in the condition with oxidative stress. In this case the cysteine residues in Keap1 are modified by electrophiles. This causes the Keap1-Nrf2 complex to collapse. Subsequently Nrf2 translocates to the nucleus and induces the transcription of phase 2 genes.

This Keap1/Nrf2 pathway has been related to different types of cancer and their therapies. Induction of Nrf2 is expected to have chemoprotective effects against ROS and chemical carcinogens [12] and mutations in Keap1 have been proven to be related to certain types of cancer [15]. Furthermore, it is thought that Keap1 could be a target in radiosensitizing tumorcells [16]. In order to develop certain drugs it is important to keep in mind the remarkable fact that every electrophile modifies a different combination of cysteine residues, called the cysteine code [15]. Electrophiles are normally not very favorable drugs, their half-life is very short because they react rapidly with different nucleophiles and they have different side effects. Electrophiles with certain patterns may for instance attack other thiol-including compounds. However getting a clearer view on these patterns

could also open new avenues to the development of drugs that modify a certain cysteine code, strong enough to disturb the Keap1-Nrf2 complex, but too weak to attack cellular compounds [13, 15]. This could be achieved by gaining more knowledge about the reactivity of the different thiols in Keap1 and other cellular compounds and their differences.

Material and methods

Thiols in reaction with 1-chloro-2,4-dinitrobenzene

In order to measure the reactivity of the thiols, we used the reaction between CDNB and thiols. This reaction is called a nucleophillic aromatic substitution via an addition-elimination principle. The thiol in this reaction represents the nucleophile and the CDNB represents the electrophile. The chloride-atom in the CDNB-molecule pulls harder on the electron pair than the carbon-atom does. Therefore the chloride will be more negatively charged and the carbon will be more positively charged. The nucleophillic sulfur will now attack this positive carbon atom thereby forming a Meisenheimer-complex wherein the carbon-atom is bound to both chloride and the sulfur group of the thiol, stabilized by the nitro-group at the ortho-position through resonance. This part of the reaction is called addition. Soon after this complex is formed the chloride will be pushed off and the conjugate of the thiol and DNB (RS-DNB) is formed (Figure 2) [17]. This conjugate can be measured at 340 nm [18]. Measuring the formation of this conjugate allows us to determine the reaction rate for different pH's and eventually translate this into the reactivity of the thiolate ion for different thiols. The thiols we used for our experiments are glutathione (pKa: 8.66) , 2-mercapto-ethanol (pKa 9.6) and 5-thio-2-nitrobenzoic acid (pKa 4.19). These are chosen considering a variety of respective pKa values.

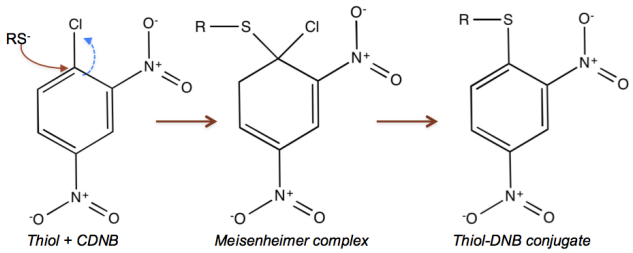

Figure 2. A schematic view of the nucleophillic aromatic substitution reaction between a thiol and 1-chloro-2,4-dinitrobenzene. The blue dashed arrow indicates the electron pair, which is localized closely to the chloride-atom. The red arrow indicates the nucleophillic attack by the sulfur atom. Molecule structures are made using Chembiodraw 14.0.

Creating a pseudo-first order reaction

The basic reaction we used was

RSH + CDNB -> RS-DNB+H^++ Cl^-

The rate constant of this reaction can be calculated using the following equation:

r=k[CDNB]*[RSH]*

Where r: reaction rate, k: rate constant, also called reaction constant, and [CDNB] & [RSH] are the concentrations of CDNB and the used thiol respectively. This equation makes it hard to calculate the rate constant because you would have to know both the CDNB and RSH concentration for each time point. This could be overcome by calculating the concentrations, which is not very precise. Therefore a pseudo-first order reaction was created by adding the CDNB in excess. This causes the concentration of CDNB to be relatively constant. Which makes it possible to simplify the equation mentioned above to the following equation:

r=k^'[RSH]*

Where *k^'=k*[CDNB]o*

This setup allows us to determine the reaction constant by simply measuring RS-DNB for different concentrations of RSH [19].

The measured reaction rates were converted into k' and eventually corrected for the concentration CDNB thereby giving us the reaction constant k. These rate constants were different for the different pH's that we performed the measurements at. Plotting these rate constants against their pH would give a logarithmic curve, which is hard to interpret. Therefore we converted the pH into the fraction of deprotonated thiol, which can be calculated using the Henderson-Hasselbalch formula. These results are shown in figure 3-5. In figure 3 we see the reaction constant k of the reaction between glutathione and CDNB for different pHs, plotted against the fraction of deprotonated GSH present at each pH. In figure 4, the reaction constant k of the reaction between TNB and CDNB is plotted against the fraction deprotonated TNB. In figure 5, the reaction constant k of the reaction between BME and CDNB is plotted against the fraction deprotonated TNB.

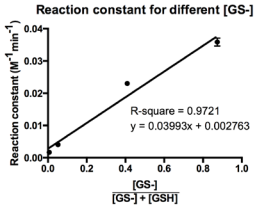

Figure 3. The rate constant of the reaction between GSH and CDNB against the fraction of deprotonated GSH, calculated using the Henderson-Hasselbalch equation. The data are shown as mean ± SD (n = 3).

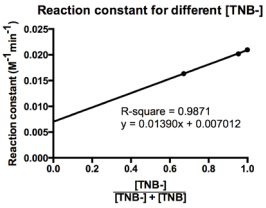

Figure 4. The reaction constant plotted against the fraction of deprotonated TNB, calculated with the Henderson-Hasselbalch equation. The data are shown as mean ± SD (n = 3).

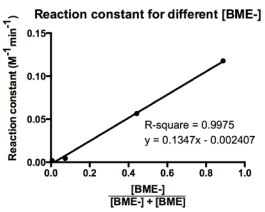

Figure 5. The rate constant of the reaction between BME and CDNB against the fraction of deprotonated BME, calculated using the Henderson-Hasselbalch equation. The data are shown as mean ± SD (n = 3).

The slope of these graphs gives us the reaction constant per fraction thiolate ion and can therefore be seen as the reactivity of the thiolate ion.

Considering the pKa value's of the thiols: TNB: 4.19; Glutathione: 8.66; and BME: 9.6 and the reactivity of their thiolate: TNB: 0.01390 $M^{-1}min^{-1}$; Glutathione: 0.03993 $M^{-1}min^{-1}$; and BME: 0.1347 $M^{-1}min^{-1}$. We see an increase in reactivity of the thiolate ion together with an increasing pKa value.

Discussion/Conclusion

Overall we see that there is indeed a paradoxal influence of the pKa value on the reactivity of the thiols, as shown in figure 6. Here we see a decrease in fraction deprotonated thiol as pKa increases but an increase in reactivity of the thiolate as pKa increases. We wanted to know what the exact influences were of these both mechanisms. This could be integrated by multiplying the fraction of deprotonated thiol with the reactivity of the thiolate ion. This was done for different pH's and plotted in figure 7. From this figure it becomes clear that thiols with a low pKa are not always more reactive. For instance TNB is the most reactive substance at pH's until 8.2. Above these pH's it is apparently better to use a thiol with a higher pKa, whose deprotonated concentration might be lower, but eventually the higher reactivity of the thiolate ion compensates for this. Therefore it is not valid to say that a low pKa leads to a higher reactivity, since this is dependent on the pH that it will be used at. Furthermore we can conclude that neither the deprotonation nor the intrinsic reactivity of the sulfur has the biggest contribution to reactivity, apparently it is dependent on the pH of the environment what weighs the heaviest. Eventually the intrinsic reactivity determines the maximum reactivity of the thiol, but this can be greatly reduced by low

fractions of thiolate ion. Therefore it can not be said what the best pKa is with respect to reactivity, but it is certain that this paradox should be taken into consideration before selecting certain thiols based on their expected reactivity.

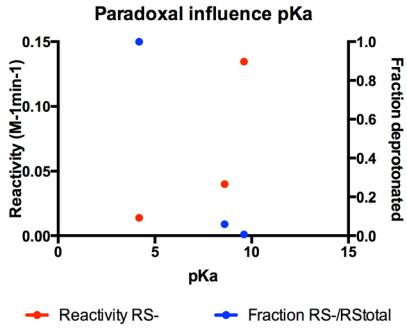

Figure 6. In blue we see the fraction of RS^- at pH 7.4, plotted on the right y-axis against pKa-value of the different thiols. In red we see the reactivity of the RS^- also plotted against the pKa of the thiols, but on the left y-axis.

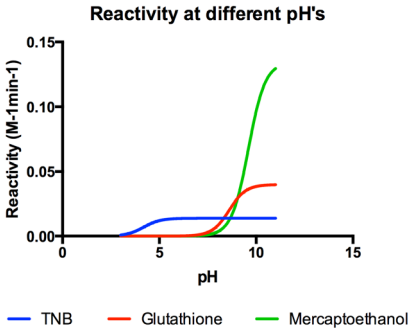

Figure 7. The reactivity of the different thiols are plotted against pH. These values are the reactivities corrected for the fraction of RS^- present at different pH's.

Role of the student

This paper is a summary of the original thesis by Daniëlle Bianchi. Dr. Guido Haenen proposed the original idea of *"the paradoxical influence of the pKa value on the reactivity of thiols"*. He assigned this problem to Danielle Bianchi as a bachelor student in BioMedical Sciences. Both Danielle and Guido together with Prof. Dr. Aalt Bast have discussed a lot about different approaches to answer the research question. The student carried out the experiments. The student under supervision of Aalt Bast did the interpretation and explanation of the results. The student wrote the thesis.

Acknowledgments

First of all I would like to thank Dr. Guido Haenen for assigning this very interesting subject to me and giving me the opportunity to carry out my own research independently, for motivating me to solve the problems I came across but at the same time for giving me the right amount of help when needed. Furthermore I would also like to thank Prof. Dr. Aalt Bast for the very interesting and often frustrating discussions we've had about solutions we both had trouble finding. You both have greatly motivated me to come up with solutions and interpretations myself and learned me a lot about research and

the scientific world. During this internship I have surely grown a lot as a student, but most of all as a researcher. I also would like to acknowledge Roger Bartholomé for his technical support, Gertjan den Hartog for his chemical knowledge and his assistance in my understanding hereof and furthermore the rest of the department of Toxicology who have all contributed to my thesis in some way. Thank you.

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