JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by American Chemical Society

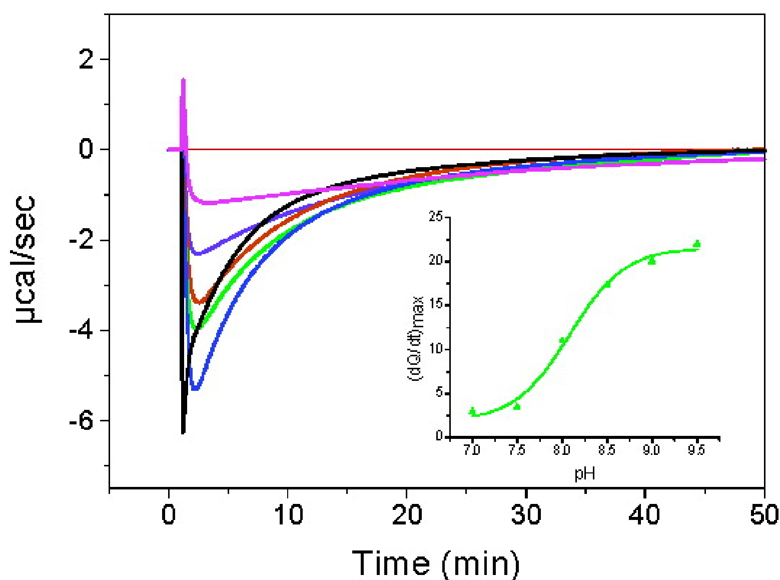
Communication

Direct Determination of Thiol p*K* by Isothermal Titration Microcalorimetry

Stephen G. Tajc, Blanton S. Tolbert, Ravi Basavappa, and Benjamin L. Miller

J. Am. Chem. Soc., **2004**, 126 (34), 10508-10509• DOI: 10.1021/ja047929u • Publication Date (Web): 06 August 2004

Downloaded from http://pubs.acs.org on April 1, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 3 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S COMMUNICATIONS

Published on Web 08/06/2004

Direct Determination of Thiol pK_a by Isothermal Titration Microcalorimetry

Stephen G. Tajc,[†] Blanton S. Tolbert,[†] Ravi Basavappa,[*,†] and Benjamin L. Miller[*,†,‡]

Department of Biochemistry and Biophysics and Department of Dermatology, University of Rochester, Rochester, New York 14642

Received April 9, 2004; E-mail: benjamin_miller@futurehealth.rochester.edu; ravi_basavappa@urmc.rochester.edu

The pK_a values of ionizable groups in molecules are critical determinants of their structure and activity. However, reliable determination of the pK_a is often difficult. Traditional methods used to determine pK_a values rely on spectroscopy or potentiometry. Since molecules tend to have a solubility minimum at their pK_a, this limitation undermines measurement at precisely those conditions that are of most interest. Under some circumstances, mixed solvent systems can be employed, but this can require a laborious search for appropriate solubilizing conditions and requires mathematical extrapolation of the data to zero organic solvent content for the estimation of aqueous phase pK_a.[1] The spectroscopic approach to pK_a measurement is usually performed by UV absorption or NMR spectroscopy. The former technique requires the presence of a chromophoric center, while the latter requires expensive and sophisticated instrumentation and is labor intensive. An alternative, more general method for pK_a determination clearly is needed. To that end, we describe herein the novel use of isothermal titration calorimetry (ITC) to accurately and reproducibly measure pK_a values of small molecules.

Since its inception, ITC has become a popular method for definitively measuring equilibrium constants (K_{eq}) and associated thermodynamic parameters and more recently has been used in the study of enzyme kinetics.[2] The basic premise of ITC is to monitor the time derivative heat change resulting from a chemical event such as ligand binding, a conformational change, dissolution, bond formation/cleavage, and ionization. Integration of the resulting data with respect to time allows the generation of the isotherm for the reaction and extraction of full thermodynamic parameters for the system. A major advantage of ITC is that it is not limited to soluble systems but is equally effective in monitoring reaction heats in suspensions.

Due to its ability to monitor heats of ionization, ITC has been used to infer pK_a values of reactive residues of enzyme:substrate complexes by monitoring the $\Delta H_{binding}$ as a function of pH and buffer composition.[3] This approach is indirect and requires the utilization of multiple buffering systems with varied enthalpies of ionization. Comparable to measuring $\Delta H_{binding}$ as a function of pH, other researchers have exploited the pH dependence of the association constant, K_a.[4] This approach is likewise not a direct measurement of the pK_a value of a particular group but is rather a global effect of multiple ionizable species on the binding affinity.

Our approach uses ITC to monitor directly the covalent reaction of a specific reagent with an ionizable group as a function of pH. To test our approach, we used thiol as the ionizable group since thiols are of tremendous importance in pharmaceutical chemistry and since their pK_a values can vary dramatically depending on their particular chemical environment. For the thiol-specific reagent, we used iodoacetamide,[5] which reacts with the ionized (thiolate) form of the thiol to produce a thioether.[6] This approach avoids the inherent limitations associated with spectroscopic techniques and in practice should be extendable to low-solubility systems.

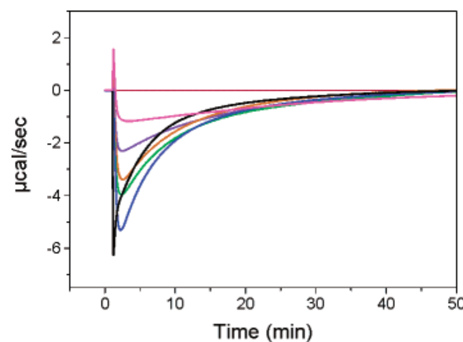


Figure 1. Calorimetric thermograms for the injection of 1.0 μL of 300 mM iodoacetamide into 250 μM L-cysteine at varying pHs (9.5, 9.0, 8.5, 8.0, 7.5, 7.0 from the lower to upper curves).

To determine pK_a, we used the ITC instrument in single-injection mode. In this mode, sufficient iodoacetamide is introduced into the sample cell in a single injection to saturate all thiolate groups. The single-injection method is often used for kinetic analysis with ITC.[2] Analysis is straightforward since the time derivative of heat change (dQ/dt) is proportional to the reaction rate. The maximum absolute value of dQ/dt is then proportional to the initial reaction rate, which due to the law of mass action is proportional to the initial concentration of thiolate.

To test this approach in the context of the thiol group in free cysteine, 1.0 μL of 300 mM iodoacetamide was injected into 250 μM L-cysteine at pH values ranging from 7.00 to 9.50 in a triple-buffer system consisting of 50.0 mM AMPSO, 50.0 mM NaP, and 50.0 mM NaPP[7] (Figure 1). The maximum absolute value of dQ/dt, denoted as $(dQ/dt)_{max}$, was measured for each pH value. These values were corrected for dilution and buffer ionization effects by subtracting the $(dQ/dt)_{max}$ obtained in the absence of cysteine in the sample cell. The corrected $(dQ/dt)_{max}$ values produce a classic titration profile when plotted as a function of pH (Figure 2). The experiment was repeated using cysteine concentrations of 400 and 500 μM. These also yielded the expected titration profile. The pK_a value can be calculated using eq 1, in which $(dQ/dt)_{max,low}$ and $(dQ/dt)_{max,high}$ are the asymptotic values of $(dQ/dt)_{max}$ at low and high pH values, respectively. This equation follows directly from the Henderson−Hasselbach relation. Nonlinear least-squares fitting of the data at the three different cysteine concentrations to this equation yields a pK_a value of 8.22 ± 0.16, which is in very good agreement with the range of pK_a values 8.3 ± 0.2 reported in the literature.[8]

$$(dQ/dt)_{max,obs} = (dQ/dt)_{max,low} + \frac{(dQ/dt)_{max,high} - (dQ/dt)_{max,low}}{1 + 10^{(pK_a - pH)}} \quad (1)$$

[†] Department of Biochemistry and Biophysics.
[‡] Department of Dermatology.

10.1021/ja047929u CCC: $27.50 © 2004 American Chemical Society

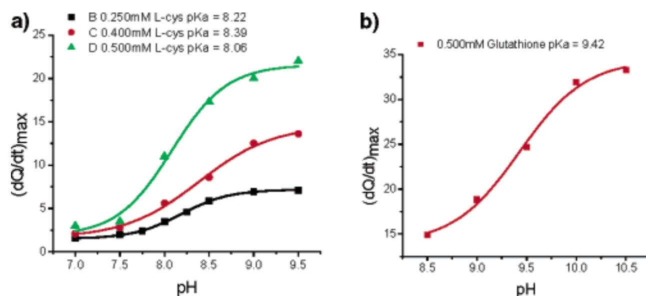


Figure 2. (a) ITC results for the injection of iodoacetamide into L-cysteine at various concentrations (250, 400, and 500 μM, from the lower to upper curves). (b) ITC result of the injection of 1.0 μL of 500 mM iodoacetamide into 500 μM glutathione at various pH ranges.

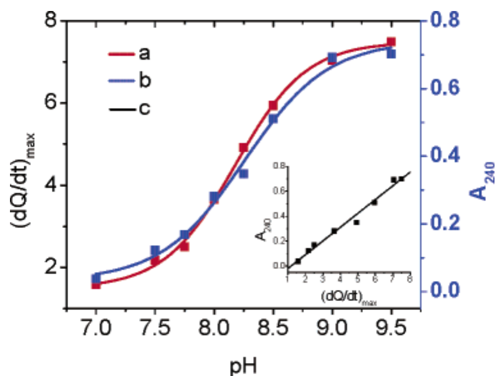


Figure 3. (a) Plot of 250 μM L-cysteine absolute $(dQ/dt)_{max}$ vs pH measured by ITC, fit to eq 1. (b) Plot of 250 μM L-cysteine vs pH measured by UV A_{240}, fit to a sigmoidal curve. (c) Plot of A_{240} vs $(dQ/dt)_{max}$; $R = 0.992$.

To corroborate our results, we determined the pK_a of identical samples of cysteine at 250 μM using an independent method, based on the differential absorption of light by the thiolate versus the thiol at 240 nm.[9] Comparison of the thiolate concentration as a function of pH using ITC and absorption at 240 nm gives titration profiles that are in excellent agreement (Figure 3; pK_a = 8.22 by ITC, 8.26 by A_{240}). Importantly, the relationship between $(dQ/dt)_{max}$ and thiolate concentration (as measured by A_{240}) is linear (Figure 3, inset), which experimentally validates our assertion that $(dQ/dt)_{max}$ and the thiolate concentration are linearly proportional.

To provide an additional demonstration of this method we employed ITC to determine the pK_a of glutathione (L-γ-glutamyl-L-cysteinylglycine). The pK_a of the central cysteine residue of glutathione has been reported to be 9.2 $\pm$ 0.15.[10] Using the same methodology described for the L-cysteine experiments, a 1.0 μL injection of 500 mM iodoacetamide was titrated into 500 μM glutathione in a pH range of 8.00 to 10.50 in the triple-buffer system. After subtracting the heats of dilution and ionization from the control experiments, the cysteine residue on the glutathione was found to have a pK_a of 9.42 $\pm$ 0.17 via ITC (Figure 2b). Like cysteine, the glutathione pK_a was also found to be independent of concentration.

Finally, to verify that this method would be suitable for pK_a determinations in proteins, we examined the human mitotic-specific ubiquitin-conjugating enzyme UbcH10.[11] UbcH10 has two cysteine residues, one at the active site (C114) with a presumably low pK_a and a buried cysteine (C102). We targeted the latter residue, titrating the protein through a pH range of 8.5 to 11.2. As shown in Figure 4, the ITC method allows us to establish a lower bound for the C102 thiol pK_a of 10.75. Measurement at pH much above 11.2 is difficult due to protein denaturation. The high pK_a value of the buried cysteine is expected due to its low dielectric environment and is in good agreement with that of the buried cysteine (C35) in thioredoxin.[12]

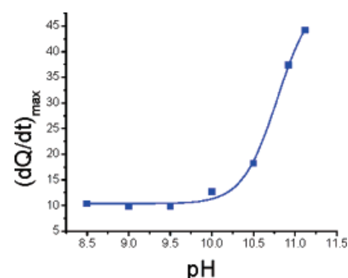


Figure 4. pK_a determination for C102 of UbcH10.

We have shown that determining thiol pK_a values via ITC is practical and straightforward for a variety of molecules from small molecules to oligopeptides to proteins. In the case of proteins with multiple cysteine residues, the cysteine residues of catalytic importance and hence functional interest typically have pK_a values that are lower than that for free thiol.[13] Thus, deconvolution of the titration of several thiols to yield the pK_a value of interest should be straightforward.

In conclusion, we have demonstrated a novel application of ITC to the measurement of pK_a values. This approach extends the conventional use of ITC into a new regime that would be beneficial for many researchers in diverse areas, including organic synthesis, drug development, and protein functional characterization. Extension of this technique to other ionizable groups should be feasible, and efforts in that direction are in progress.

Acknowledgment. ITC instrument used in this study was purchased through a grant from the NIH-NIGMS, #5-R01-GM-062825-03. R.B. is a Research Scholar of the Leukemia and Lymphoma Society. The authors thank Charles Mace, Rahul Tyagi, and Jessica Snyder for assistance with the preparation of UbcH10.

Supporting Information Available: Full description of procedures and treatment of data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Avdeef, A.; Box, K. J.; Comer, J. E.; Gilges, M.; Hadley, M.; Hilbert, C.; Patterson, W.; Tam, K. Y. *J. Pharm. Biomed. Anal.* **1999**, *20*, 631−641.
(2) Williams, B. A.; Toone, E. J. *J. Org. Chem.* **1993**, *58*, 3507−3510.
(3) Subramanian, S.; Ross, P. D. *J. Biol. Chem.* **1979**, *254*, 7826−7830.
(4) Xie, D.; Gulnik, S.; Collins, L.; Gustchina, E.; Suvorov, L.; Erickson, J. W. *Biochemistry* **1997**, *36*, 16166−16172.
(5) Post, P. L.; Trybus, K. M.; Taylor, D. L. *J. Biol. Chem.* **1994**, *269*, 12880−12887.
(6) Witkowski, A.; Joshi, A. K.; Smith, S. *Biochemistry* **2002**, *41*, 10877−10887.
(7) Schlegal, B. P.; Jez, J. M.; Penning, T. M. *Biochemistry* **1998**, *37*, 3538−3548.
(8) Krekel, F.; Samland, A. K.; Macheroux, P.; Amrhein, N.; Evans, J. N. S. *Biochemistry* **2000**, *39*, 12671−12677.
(9) Creighton, T. E.; Kortemme, T. *J. Mol. Biol.* **1995**, *253*, 799−812.
(10) Tang, S. S.; Chang, G. G. *J. Biochem.* **1996**, *119*, 1182−1188.
(11) Lin, Y.; Hwang, W. C.; Basavappa, R. *J. Biol. Chem.* **2002**, *24*, 21913−21921.
(12) Chivers, P. T.; Prehooda, K. E.; Volkman, B. F.; Kim, B.-M.; Markley, J. L.; Raines, R. T. *Biochemistry* **1997**, *36*, 14985−14991.
(13) Guengerich, F. P.; Fang, Q.; Liu, L.; Hachey, D. L.; Pegg, A. E. *Biochemistry* **2003**, *42*, 10965−10970.

JA047929U