Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 610-615

A GUANIDINE HYDROCHLORIDE INDUCED CHANGE IN RIBONUCLEASE WITHOUT GROSS UNFOLDING

Joyce F. Miller and D. W. Bolen

Dept. of Chemistry and Biochemistry
Southern Illinois University
Carbondale, IL 62901

Received February 14,1978

SUMMARY: Although denaturation of ribonuclease by guanidine hydrochloride to a random coil has been considered to be a simple two-state mechanism, the time dependence of our calorimetric data indicate that a cooperative endothermic pretransition may occur near 1.25 M. guanidine hydrochloride (pH 6 and 25°C) without gross unfolding of the protein. Reexamination of other observables as a function of guanidine hydrochloride concentrations as well as activity measurements suggests the possibility of some process other than simple binding occurring in the concentration range below the onset of gross denaturation.

A variety of measurements (ORD, CD, difference spectra, viscosity, and NMR) have been made on the ribonuclease (RNase)* -guanidine hydrochloride (GdnHCl) system to follow protein unfolding. Evidence has been given by Salahuddin and Tanford (1) to indicate that denaturation is two-state (native and denatured) and that no intermediate states exist in the transition region for this system. However, NMR (2) and specific volume (3) data suggest some changes occur in the native state of the protein at GdnHCl concentrations which are lower than required for the onset of gross protein unfolding.

We originally set out to calorimetrically measure the enthalpy change for RNase unfolding but found a time dependence in our measurements at GdnHCl concentrations in which only the native state should exist. Since this would greatly affect the interpretation of our calorimetric results it was necessary to investigate the nature of this time dependence.

PROCEDURES

Ribonuclease A, Sigma Type IA (Lot 65C-0172) and IIA (56C-8020) was purified by the method outlined by Taborsky (4) except that the "D" fraction was concentrated by an Amicon Diaflo Ultrafiltration UM-2 membrane to a volume of 60-100 ml and applied to a G-25 Sephadex column equilibrated with 10^{-3} M HCl. The peak fractions were lyophilized and stored at 4°C. The activity was meas-

*Abbreviations: PIPES -Piperazine-N-N'-bis[2-ethane sulfonic acid]; RNase-ribonuclease; GdnHCl-Guanidine hydrochloride.

0006-291X/78/0812-0610$01.00/0

ured with Cytidine 2':3' cyclic monophosphate by the method of Crook et al. (5) and at 285 nm gave an initial velocity greater than 2 x 10^{-3} ΔOD min^{-1} per µg/ml of RNase. The concentration of RNase was determined spectrophotometrically at 277.5 nm (pH = 6) using ε of 9800 l $Mole^{-1}$ cm^{-1} (6).

Guanidine Hydrochloride (GdnHCl) was prepared by the method outlined by Nozaki (7) from guanidine carbonate. The melting point was 187°-188° and the absorbance of a 6 M solution was less than 0.10 at 230 nm. Solutions were treated with activated charcoal, filtered, and concentrations were determined by refractive index difference using the equation of Nozaki (7).

PIPES buffer (Sigma) 0.05 M in 0.126 M NaCl (initial ionic strength of 0.2) was prepared and titrated to pH 6.00 with a Beckman pH Meter fitted with a Markson MiraMark 800 combination electrode. GdnHCl was dissolved in the pH 6 buffer and the pH recorded. RNase was dissolved in buffer at 5-6 mg/ml and the pH adjusted to 6.00.

The calorimetric measurements were made on a Picker Dynamic Flow Microcalorimetre (8) maintained at 25.000°C with a Sodev Programmable Circulating Thermostat. Reagents were delivered with a bent-piston pump with a diaphragm delivery device (9) used for delivering the protein. The GdnHCl was pumped against buffer to obtain a baseline and the endothermic heat of dilution was compensated by adding heat electrically to the system. When a new baseline was established, the buffer was switched to RNase solution and the steady state signal generated. The volume of the cell was given as 0.2 ml and the flow rates, which were measured each time, varied from 0.12 to 0.3 ml/min. Dilution was 1:1 in all cases.

The effluent from the sample cell was returned to the reference cell to maintain the same heat capacity in both cells. The signal was calibrated electrically under the same conditions as the measurement.

Fractions of the effluent were collected and the final concentrations of GdnHCl and RNase determined. The heat of dilution of the enzyme was determined each time and found to be insignificant.

The difference spectra were obtained on a Beckman ACTA MVI Spectrophotometer using PIPES buffer 0.05 M, 0.126 M NaCl pH 6, at 287.5 nm. Appropriate GdnHCl concentrations were prepared and pH recorded. Semi-micro quartz cuvettes with 1 cm path length were used with final RNase concentrations of approximately 0.3 mg/ml. Equilibrium values and kinetic data were obtained from the same experiment. The sample compartment was maintained at 25.0 degrees by a Sodev Programmable Circulating Thermostat.

RESULTS

The results of difference spectra given in Figure 1 indicate a sharp transition between 1.8 M and 4 M with a midpoint at 2.9 M GdnHCl. These data, generally interpreted in terms of a two-state mechanism, represent gross protein unfolding and are consistent with the results of Salahuddin and Tanford (1), Greene and Pace (10), and Puett (11).

The kinetics of the denaturation process (Figure 1) are typical of unfolding with the lowest rate constants near the midpoint (12). The reaction rates below 2.0 M GdnHCl can not be accurately determined since the observed spectral difference ($\Delta\varepsilon$) becomes very small. However, the trend in the rate

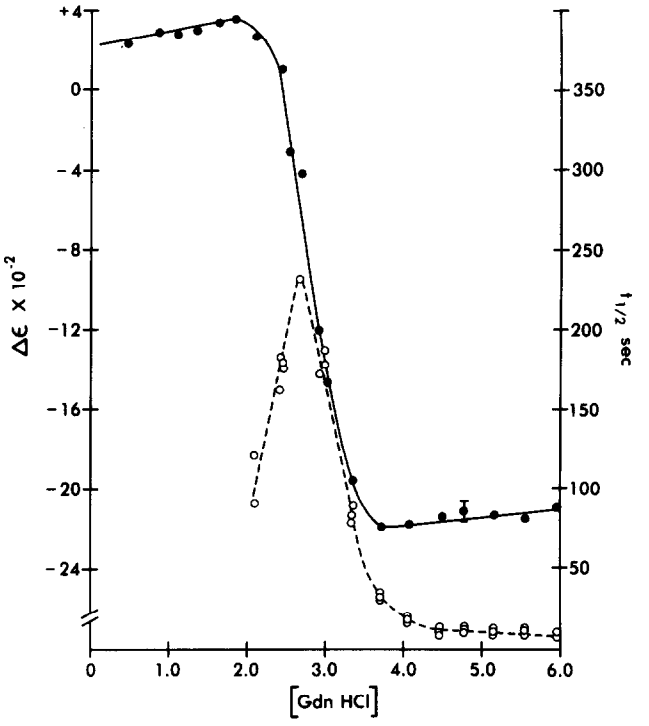

Figure 1: Difference Spectra of RNase in 0.05 M PIPES, 0.126 M NaCl as a Function of GdnHCl Concentration. RNase 0.3 to 0.6 mg/ml, 1.0 cm cell. Left Ordinate - Equilibrium values, ($\Delta\varepsilon$) ●; Error bar represents typical standard deviation in each point. Right Ordinate - half life of protein unfolding, O.

constants suggests the rate of unfolding is increasing at 1.8 M GdnHCl, while the extent of unfolding is barely detectable.

It is necessary for the rate of a reaction to be reasonably rapid if flow calorimetry is to be used to measure the enthalpy change. If the reaction is not complete during the time the mixed reactants are in contact with the heat detector, the magnitude of the steady state signal will be less than the true value. In the absence of kinetic data from an independent measurement it is common practice to confirm that a reaction is complete by measuring the reaction heat at two different flow rates (different resident times). If the enthalpy changes are identical, one can conclude that the reaction is complete at the higher flow rate. We used resident times of about 45 and 80 seconds in these experiments to check on reaction completion.

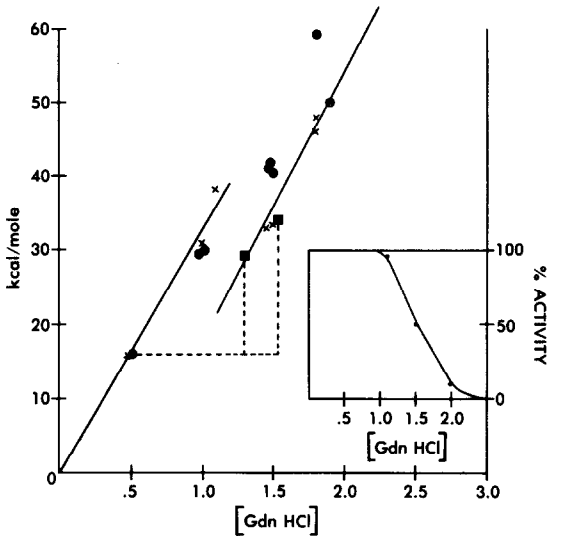

Figure 2: Calorimetric Data of RNase in 0.05 M PIPES, 0.126 M NaCl as a Function of GdnHCl Concentration. Longer resident times, x (72 - 85 sec); shorter resident times ● (0 - 1.2 M are ∿ 40 sec; 1.2 to 1.8 M are ∿ 50 sec); resident time of 103 sec ⊗ ; Chord measurement, RNase in 0.5 M GdnHCl initially (50 sec) ■ . Final RNase concentration was 2.5 - 3 mg/ml. Insert: Ratio of RNase activity in GdnHCl to activity in equimolar NaCl, Sela et al. (6).

With these resident times we confirmed that the RNase-GdnHCl interactions were complete when final GdnHCl concentrations were in the range from 0 to 1.2 M. However, between 1.2 M and 1.9 M GdnHCl a time dependence was observed (Figure 2). Contrary to expectation, the interaction enthalpy changes measured at the slower flow rates (longer resident times) were lower than those at faster flow rates.

The enthalpy changes determined from longer resident times are more difficult to obtain because of mixing inefficiency and the very high background enthalpy change of GdnHCl dilution. Chord measurements provide some advantages in overcoming these problems and were performed by mixing RNase dissolved in 0.5 M GdnHCl with sufficient GdnHCl concentration to give 1.3 M and 1.55 M final GdnHCl. It can be seen (Figure 2) that these measurements are consistent with the lower heats of RNase-GdnHCl interaction at the longer resident times in the 1.2 to 1.9 M region.

Two measurements, made under the same conditions (except for final GdnHCl concentration), yielded a higher enthalpy change for 1.18 M than for 1.45 M GdnHCl with both experiments having a 71 second resident time. The value at 1.18 M falls on the same line as values at .5 M and 1.0 M.

A measurement at 1.9 M at 103 second resident time indicates that the reaction was complete at 80 seconds.

DISCUSSION

The primary observation in these experiments is that the interaction of GdnHCl with RNase is time dependent when GdnHCl concentrations between 1.2 and 1.9 M are used. Within this range of GdnHCl concentrations it is clear that the protein-GdnHCl interaction is not complete in 45 seconds, though it appears to be complete in 70 to 80 seconds. The rather abrupt change in resident time-dependence occurs at GdnHCl concentrations beyond about 1.25 M and appears to be a cooperative endothermic process.

There is a mounting body of evidence to suggest that several local changes occur in RNase in the range of 0 - 1.9 M GdnHCl (2, 3, 10, 11). A reexamination of published ORD (10) and CD (11) data suggests irregularities in the 1.0 to 1.5 M GdnHCl region though the deviations have been regarded as within experimental error. Benz and Roberts (2) found NMR histidine $C_{(2)}$-H resonance changes occuring at GdnHCl concentrations less than that necessary for gross unfolding (1.8 M) and suggested local structural changes near the RNase active site.

Activity measurements have provided the clearest expression of cooperative changes occuring at GdnHCl concentrations below the onset of major unfolding. The data of Sela et al. (5) indicate that loss in enzymatic activity occurs at GdnHCl concentrations much less than that required for unfolding. Activity of RNase in GdnHCl relative to that in equivalent NaCl concentrations (to correct for ionic strength effects) indicate a relatively sharp reduction in the ratio of activity between 1.2 and 1.8 M GdnHCl (9). Their data (included as an inset of Figure 2) are seen to correlate with the effect we have observed by flow microcalorimetry.

The data suggest that the endothermic enthalpy change is approximately 17 kcal/mole. However, if protons are released or taken up in a system, the measured enthalpy change is a function of the ionization enthalpy of the buffer. Because of the probable proton change and the kinetics for the transition, the 17 kcal/mole cannot be interpreted as the true enthalpy change, nor can statements be made about the specific nature of the transition from the calorimetric data. Nevertheless, the nature of calorimetric measurements is to give an algebraic sum of all enthalpy contributions accompanying a process and in this case, the net enthalpy changes appear to be more sensitive to molecular events than the usual observables for monitoring structural change.

Acknowledgement. Support of this research by the National Institutes of Health (Grant GM-22330) is gratefully acknowledged.

BIBLIOGRAPHY

1. Salahuddin, Ahmad, & Tanford, Charles (1970) Biochemistry 9, 1342-1347
2. Benz, F. W., & Roberts, G. D. K. (1975) J. Mol. Biol. 91, 345-365
3. Crouch, T. H., & Kupke, D. W. (1977) Biochemistry 16, 2586-2593
4. Taborsky, George (1959) J. Biol. Chem. 234, 2652-2656
5. Crook, E., Mathias, A. P. & Rabin, B. R. (1969) Biochem. J. 74, 234-238
6. Sela, M., Anfinsen, C., & Harrington, W. (1957) Biochim. Biophys. Acta. 26, 502-512
7. Nozaki, Yasuhiko (1972) Methods in Enzymology XXVI (Part C) C.H.W. Hirs and Serge Timasheff, ed., 43-50
8. Picker, Patrick (1974) Canadian Research and Development 11-16
9. Picker, P., Miller, J. F., & Bolen, D. W. (1978) Anal. Biochem. (in press)
10. Greene, R. F., & Pace, C. N. (1974) J. Biol. Chem. 249, 5388-5395
11. Puett, David (1972) Biochemistry 11, 1980-1990
12. Tanford, Charles (1969) Adv. in Prot. Chem. 23, 121-282