THERMAL DENATURATION OF LOBSTER HEMOCYANIN

JOSEPH H. FLEISHER*

Marine Biological Laboratory, Woods Hole, Mass. (U.S.A.)

INTRODUCTION

A survey of the literature on hemocyanins reveals that little or no work has been done on the kinetics of heat denaturation of these compounds. The recent publication of a convenient method for the purification of lobster hemocyanin[1] provided the means by which an adequate amount of the purified protein could be prepared for the kinetic studies described below.

In the study to follow, no distinction has been made in the determination of the concentration of soluble protein between soluble intact hemocyanin and soluble dissociation products of this molecule which may occur by reason of changes in pH[2].

EXPERIMENTAL

Procedure

Details of the purification are given in reference[1]. The pellets of hemocyanin obtained by high speed centrifugation were extracted with 0.02 *M* acetate buffer. Portions of the extract were adjusted to a final pH of 7.40 with 0.5 *M* phosphate buffer, to pH 8.48 with 0.5 *M* tris(hydroxymethyl)aminomethane (tris buffer); and to pH 9.16 with 0.5 *M* glycine-sodium hydroxide buffer. Final molarities were 0.05 *M* for all three and ionic strength was maintained at 0.13 by the addition of appropriate quantities of NaCl.

For studying the rate of thermal denaturation, 0.05 to 0.15 ml portions of the freshly prepared solutions were placed in pieces of thin glass tubing about 4 inches in length and 2 mm inside diameter. One end of the tubing was then sealed with a gas-oxygen flame. After cooling, the preparation was immersed in a water bath held at the temperatures noted in Table I $\pm$ 0.05° C. Tubes containing the heated hemocyanin were withdrawn at the time intervals noted in Table I and immediately cooled by immersion in ice water. Under these conditions, the denatured protein precipitates even before cooling as an amorphous white solid. The latter was separated from the remaining soluble protein by centrifugation at 2,500 r.p.m., for 10 minutes. The concentration of the soluble protein remaining in solution was determined by means of the specific refractive increment[3].

Presentation of data

The ratio of the specific refractive increment, n_t, after known times of heating to the total (maximal) change of the specific refractive increment obtained by heating for 10 minutes in a water bath at 100° C, n_0, was found to be proportional to the change in concentration of protein remaining in solution. Consequently the logarithm of $n_t/n_0 = -\log c$ where c = concentration. When the logarithm of n_t/n_0 (% specific refractive increment) was plotted against time for each of the temperatures studied,

* Present address: The Biochemical Branch, Chemical Warfare Laboratories, Army Chemical Center, Maryland.

References p. 33.

straight lines were obtained, indicating that the denaturation followed first order kinetics. The specific rate constants were evaluated by multiplying the slope of each of the plots by 2.303, making use of the formula below:

$$-\log \frac{n_t}{n_0} = \left(\frac{k}{2.303}\right) t + \text{constant}$$

A representative plot for each pH is shown in Fig. 1 and the velocity constants are enumerated in Table I. All rates were found to be reproducible in repeated determinations with the same preparation.

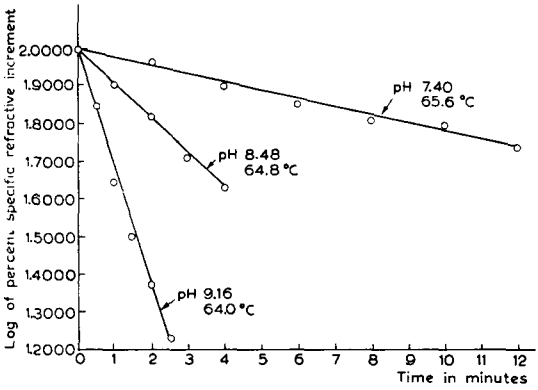

Fig. 1. Rate of denaturation (change in specific refractive increment) at various temperatures and pH.

TABLE I

HEAT DENATURATION OF LOBSTER HEMOCYANIN UNDER VARIOUS CONDITIONS

pH of incubation mixture at room temperature	*Temperature used for denaturation* °C	*Time* (minutes)	*Specific refractive increment* n_t	*Residual soluble protein* $\frac{n_t}{n_0} \times 100$ (percent)	*k (first-order)* min^{-1}
7.40	65.6	0	0.0149	100.0	0.05
		2	0.0136	91.3	
		4	0.0120	80.4	
		6	0.0107	71.8	
		8	0.0096	64.4	
		10	0.0091	61.1	
7.40	69.6	0	0.0151	100.0	0.24
		1	0.0116	76.8	
		2	0.0097	64.2	
		3	0.0075	49.6	
		4	0.0061	40.4	
		5	0.0041	27.2	
7.40	71.5	0	0.0151	100.0	0.52
		1	0.0080	53.0	
		2	0.0049	32.5	
		3	0.0034	22.5	
		4	0.0023	15.2	
		5	0.0011	7.3	
7.40	73.6	0	0.0148	100.0	1.80
		½	0.0060	40.5	
		1	0.0027	18.3	
		1½	0.0010	6.8	
8.48	63.0	0	0.0123	100.0	0.10
		3	0.0092	73.2	
		6	0.0067	53.6	
		9	0.0053	42.3	
		12	0.0037	30.2	

(Continued on following page)

References p. 33.

(continuation of TABLE I)

pH of incubation mixture at room temperature	*Temperature used for denaturation* °C	*Time* (minutes)	*Specific refractive increment* n_t	*Residual soluble protein* $\frac{n_t}{n_0} \times 100$ (percent)	k (first order) min^{-1}
8.48	64.8	0	0.0123	100.0	0.22
		1	0.0099	80.4	
		2	0.0080	65.0	
		3	0.0063	51.2	
		4	0.0052	42.3	
8.48	66.9	0	0.0123	100.0	0.51
		½	0.0094	76.5	
		1	0.0076	61.7	
		1½	0.0058	47.2	
		2	0.0048	39.0	
8.48	68.8	0	0.0123	100.0	1.20
		½	0.0064	52.0	
		1	0.0039	31.7	
		1½	0.0021	17.1	
		2	0.0012	9.8	
9.16	58	0	0.0136	100.0	0.05
		5	0.0108	79.5	
		10	0.0096	70.6	
		15	0.0060	44.0	
		20	0.0044	32.3	
		25	0.0037	27.2	
9.16	60	0	0.0136	100.0	0.15
		2	0.0105	77.2	
		4	0.0077	56.6	
		6	0.0058	42.7	
		8	0.0039	28.7	
		10	0.0027	19.8	
9.16	62	0	0.0136	100.0	0.34
		1	0.0098	72.1	
		2	0.0074	54.5	
		3	0.0052	38.3	
		4	0.0034	25.0	
9.16	64	0	0.0136	100.0	0.74
		½	0.0095	69.9	
		1	0.0060	44.2	
		1½	0.0042	30.9	
		2	0.0032	23.5	
		2½	0.0023	16.9	

Effect of temperature at constant pH

The energy of activation can be evaluated directly from the first-order rate constants by means of the Arrhenius equation

$$k = Ae^{\frac{-E}{RT}}$$

or

$$\log_e k = \frac{-E}{RT} + c$$

Therefore the natural logarithms of the rate constants, k, were plotted against the

References p. 33.

reciprocals of the absolute temperatures used. From the slopes of the straight lines obtained at each pH as shown in Fig. 2, the apparent activation energy, E, was found to be $9 \cdot 10^4$ calories mol^{-1}, a value comparable to activation energies found for other protein denaturations[4].

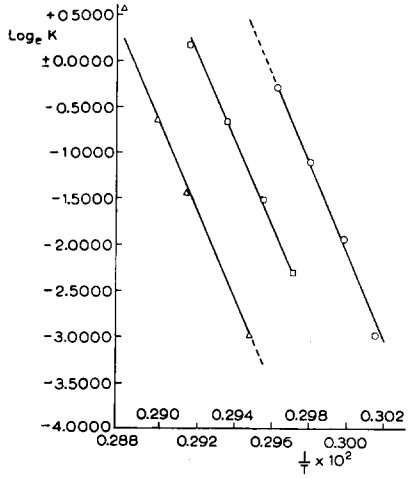

Fig. 2. Arrhenius plots relating the rate of denaturation to the reciprocal of the absolute temperature at pH 7.4, △—△; pH 8.48, □—□; and pH 9.16, ○—○.

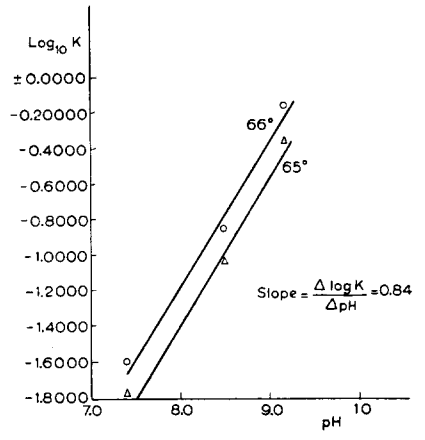

Fig. 3. Rate of thermal denaturation at varying pH values.

Effect of pH at constant temperature

Extrapolation of the temperature dependence curves in Fig. 2 permitted a comparison of $\log_{10} k$ with pH at constant temperature. Fig. 3 shows that at both 65° and 66° C, $\frac{\Delta \log_{10} k}{\Delta \text{ pH}}$ was + 0.84 probably not significantly different from + 1 over the pH range studied. This would indicate that at constant temperature, the rate of denaturation varies inversely as the first power of the hydrogen ion concentration as a reasonable approximation.

Thermodynamic formulation

GLASSTONE *et al.*[5] describe the effective rate of formation of the activated complex, c_a by the equation:

$$v = c_a \frac{KT}{h} \tag{1}$$

where
v = rate;
K = Boltzmann's constant, $1.38 \cdot 10^{-16}$ erg degree^{-1};
h = Planck's constant, $6.62 \cdot 10^{-27}$ erg second;
and T = absolute temperature.

Moreover, if k is the specific rate constant, the velocity of the forward reaction (toward formation of the activated complex) is given in the usual manner by

$$v = k\, c_{\text{reactant}} \tag{2}$$

From equations (1) and (2) it follows that $c_a \frac{KT}{h} = c_{\text{reactant}}$ and $k = \frac{c_a}{c_{\text{reactant}}} \cdot \frac{KT}{h}$

References p. 33.

but $$\frac{c_a}{c_{\text{reactant}}} = K_a; \text{ therefore } k = \frac{KT}{h} \cdot K_a \tag{3}$$

where K_a is the constant for the equilibrium which may be presumed to exist between the reactants and the activated complex. Variations in activities of the reactants have been minimized by maintaining a constant ionic strength allowing concentration of reactants to be used in calculations. Therefore the constant K_a is exactly analogous to any other equilibrium constant. It may be related to ΔF^*, ΔH^*, and ΔS^*, the standard free energy, heat content, and entropy changes, respectively, accompanying the formation of the activated state from the reactants by means of the following thermodynamic relationships.

$$\Delta F^* = -RT \ln K_a \tag{4}$$

and $$K_a = e^{\frac{-\Delta F^*}{RT}} \tag{5}$$

Substituting in (3)

$$k = \frac{KT}{h} e^{\frac{-\Delta F^*}{RT}} \tag{6}$$

also $$\Delta H^* = E - RT \tag{7}$$

and $$\Delta S^* = \frac{\Delta H^* - \Delta F^*}{T} \tag{8}$$

The logarithmic expression of equation (6) is $\Delta F^* = RT (\ln K/h + \ln T - \ln k)$

$$\Delta F^* = 4.58T (\log K/h + \log T - \log k)$$
$$\Delta F^* = 4.58T (10.318 + \log T - \log k) \tag{9}$$

ΔH^* can be obtained from the activation energy, E by equation (7) and ΔS^* can be obtained from equation (8) once the values of ΔH^* and ΔF^* are known. The values for these functions at all temperatures and pH's studied are given in Table II.

TABLE II

THERMODYNAMIC ANALOGUES OF ACTIVATION REACTIONS FOR HEAT DENATURATION OF HEMOCYANIN

pH	*Temperature °C*	*ΔF^* cal mol^{-1}*	*ΔH^*§ cal mol^{-1}*	*ΔS^* cal degree^{-1}*
7.40	65.6	22,000	80,286	172
	69.6	21.200	80,278	173
	71.5	20,400	80,275	174
	73.6	20,000	80,270	176
8.48	63.0	21,800	80,292	174
	64.8	20,900	80,288	176
	66.9	20,200	80,284	177
	68.8	20,050	80,282	176
9.16	58.0	21,400	80,302	178
	60,0	20,700	80,298	179
	62.0	20,400	80,294	179
	64.0	20,000	80,290	179

§ Corrected for the heat of dissociation of one proton (see text).

DISCUSSION

The influence of pH on the denaturation of lobster hemocyanin may be interpreted in terms of STEINHARDT's[6] concept of the importance of ionization processes in

References p. 33.

denaturation over the pH range used in this study. STEINHARDT corrected the apparent activation energy, E^*, for the thermal denaturation of pepsin for the heat of dissociation of protons from 5 critical groups. Since the heat of dissociation of a single proton was found to be 9,040 calories, the total correction was 45,200 calories per mole.

In the case of lobster hemocyanin, a plot of the logarithm of the rate constants at constant temperature against pH yields a straight line whose slope, n, equal to the number of protons involved is approximately one (0.84). At the pH values involved, the proton may be assumed as coming from the dissociation of an amino group as in the case of pepsin. Consequently, the same correction for the dissociation for one proton, 9,040 calories can be deducted from the apparent activation energy of 90,000 calories giving a corrected value of 80,960 calories.

Since a plot of ln k versus $1/T$ (Fig. 2) falls on a straight line, one would expect ΔH^* and ΔS^* to be essentially independent of temperature. Inspection of Table II shows this to be true over the range of conditions used. The high heats of activation yield a positive free energy of activation. The fact that the reaction still occurs with appreciable velocity at the temperatures studied is explained by the large increases of entropy accompanying denaturation of proteins[5] of which the entropy of activation, ΔS^*, measured in this study is but a part. It is realized that the values of ΔF^*, ΔH^* and ΔS^* given (Table II) are in reality orders of magnitude, and could be rounded off to 20,700 cal mol^{-1}, 80,000 cal mol^{-1} and 175 cal $degree^{-1}$, respectively. When this is done they are still comparable with values for these functions given by KUNITZ[6] and EISENBERG AND SCHWERT[7].

ACKNOWLEDGEMENTS

The author wishes to thank Dr. MAX LAUFFER for his advice on the experimental portion of this investigation reported as an abstract [*Biol. Bull.*, 109 (1955) 359], and Dr. HARRY MICHEL for helpful criticism of the manuscript.

SUMMARY

1. A procedure for the determination of heat denaturation of hemocyanin by means of changes in specific refractive increment has been presented. The method possesses the advantage of requiring a small volume (0.1 ml) of solution for analysis.

2. Denaturation was found to follow first order kinetics and yielded an activation energy of 80,960 cal mol^{-1}.

3. The rate of denaturation of hemocyanin at constant temperature varied inversely as the first power of the hydrogen ion concentration, suggesting a dissociation of one proton in the range of pH studied.

4. Thermodynamic analogues based upon the theory of absolute reaction rates were calculated from the data. Approximate values of ΔF^*, ΔH^*, and ΔS^* were found to be approximately 20,700 cal mol^{-1}, 80,000 cal mol^{-1} and 175 cal $degree^{-1}$, respectively.

REFERENCES

1 M. A. LAUFFER AND L. G. SWABY, *Biol. Bull.*, 108 (1955) 290–295.
2 S. BROHULT, *J. Phys. Chem.*, 51 (1947) 206–217.
3 E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, C. C. Thomas, Springfield, Ill., 1948, Chapter 25.
4 I. W. SIZER, *Advances in Enzymol.*, 3 (1943) 35.
5 S. GLASSTONE, K. J. LAIDLER AND H. EYRING, *Theory of Rate Processes*, McGraw-Hill Book Co., New York, 1941.
6 J. STEINHARDT, *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.*, 14, No. 11 (1937).
7 M. KUNITZ, *J. Gen. Physiol.*, 32 (1948) 241–263.
8 M. EISENBERG AND G. W. SCHWERT, *J. Gen. Physiol.*, 34 (1951) 583–606.

Received April 12th, 1956