THE EQUILIBRIUM BETWEEN ACTIVE NATIVE TRYPSIN AND INACTIVE DENATURED TRYPSIN

By M. L. ANSON and A. E. MIRSKY

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller Institute for Medical Research, New York)

(Accepted for publication, October 5, 1933)

Hemoglobin denatured by acid or coagulated by heat and dissolved in acid becomes native again when brought to nearly neutral solution. From this fact the conclusion was drawn that there is an equilibrium between native and denatured protein which depends on the temperature and the pH (Anson and Mirsky, 1925). If there is such an equilibrium then at a suitable pH denaturation brought about by heating should be reversed by cooling. Reversal of heat denaturation by mere cooling was actually found to take place with serum albumin, globin, and pepsin (unpublished experiments). In none of these three cases was reversal complete, so none of these three proteins was suitable for the exact study of definite equilibrium states. A suitable protein has now been found. Trypsin, which catalyzes the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). And the denaturation of trypsin is readily and completely reversible. If trypsin is heated to 60°C. in 0.05 N acid it is converted into a protein which is completely precipitable by quarter saturated ammonium sulfate. When the heated trypsin is cooled it changes back into the original form which is not precipitated by quarter saturated ammonium sulfate. If trypsin is heated or cooled to a temperature around 40°C. a definite fraction is precipitable by salt (Northrop, 1932).

Since native trypsin digests other proteins and denatured trypsin does not, the denaturation of trypsin can be followed by activity measurements. If the digestion mixture is alkaline enough and contains enough urea then there is no change of inactive denatured trypsin into active native trypsin during the measurement of tryptic

activity. The same results are obtained whether denaturation is followed by measurements of activity with the urea technique or by measurements of the formation of salt-precipitable protein. In the present investigation the urea technique alone has been used both because of its simplicity and because it can be applied even to dilute solutions of trypsin. For the definition of trypsin activity units ($[T.\ U.]^{Hb}$) and the details of the procedure a previous paper (Anson and Mirsky, 1933) should be consulted. Hemoglobin is used as the protein substrate which is digested.

TABLE I

Effect of Acid, Alkali, and Alcohol on the Equilibrium between Native and Denatured Trypsin

Composition of solvent	Temperature of inactivation	$[T.\ U.]^{Hb}$ per ml. before heating $\times 10^4$	$[T.\ U.]^{Hb}$ after heating and cooling $\times 10^4$	$[T.\ U.]^{Hb}$ when heated to temperature of inactivation $\times 10^4$	$[T.\ U.]^{Hb}$ when cooled to temperature of inactivation $\times 10^4$	Percentage inactivation
	°C.					
0.01 N HCl	44.1	21.6	21.4	11.4	11.0	48
0.01 N HCl	44.0	199.2		99.6		50
0.003 N HCl	54.5	28.8	27.6	14.7	13.2	51.5
0.001 N HCl	61.3	28.8	28.4	14.4	15.2	48.4
0.01 N HCl in 10 per cent alcohol	38.5	28.8	28.4	14.4	14.7	49.4
0.05 N NaOH	0	345.0	260.0	17.4		95.0

Existence of Equilibria.—Under any definite conditions under which there is no irreversible inactivation, a definite fraction of the trypsin is in the active, native form and a definite fraction in the inactive, denatured form. The percentage inactivation at a given temperature is the same whether the trypsin solution is heated or cooled to that temperature (*cf.* Table I). In general the percentage denaturation at equilibrium depends only on the conditions at equilibrium and is the same whether one starts with native or denatured trypsin.

Effect of Concentration.—The concentration of a trypsin solution can be varied ten times without any effect on the percentage denaturation under given conditions (*cf.* Table I). The kinetics of denaturation are therefore the same as the kinetics of the reversal of denaturation.

Effect of pH.—In a rough way the more trypsin is ionized by either acid or alkali the more the equilibrium between native and denatured trypsin is shifted toward the denatured form. A theory of the mechanism of denaturation by acid and alkali will be presented in a paper on the equilibrium between native and denatured hemoglobin. According to this theory the effect of pH on the equilibrium between the native and denatured forms of a protein must be correlated with differences in the titration curves of the native and denatured forms. The data at present available do not permit any detailed comparison of the effects of pH on the ionization and denaturation of trypsin.

The marked effect of pH on the equilibrium between native and denatured trypsin is not apparent at room temperature. At 25°C. native trypsin is the equilibrium form even in 0.01 N hydrochloric acid. That is, denatured trypsin, if brought to 25°C. in 0.01 N hydrochloric acid, changes completely into native trypsin. The importance of pH is clear at higher temperatures. If trypsin is heated at any pH a temperature is reached at which the enzyme is half denatured. This temperature of half denaturation is very sensitive to the pH and is lowered by either acid or alkali (*cf.* Table I). Only in acid solution can the experiments be carried out without irreversible inactivation. In acid solution, as is shown in Table I, the activity after heating and cooling is the same as before heating. In alkaline solutions in which the temperature of half denaturation is low there is irreversible inactivation. In neutral solutions in which the temperature of half denaturation is high, the denatured trypsin is digested so rapidly by the native trypsin that measurements of equilibria are impossible. The irreversible inactivation by alkali and the digestion in neutral solution have been studied in detail by Northrop and Kunitz (1934).

The Effect of Temperature.—In 0.01 N hydrochloric acid trypsin is almost completely native at 40°C. and almost completely denatured at a temperature 10° higher. The logarithm of the equilibrium constant (the ratio of native trypsin to denatured trypsin) is proportional to the reciprocal of the absolute temperature as is stated by van't Hoff's relation between the heat of reaction and the effect of temperature on the equilibrium

$$\ln K = -\frac{\Delta H}{KT} + C$$

Table II gives the observed values of the percentage denaturation and the values calculated from van't Hoff's equation, assuming the heat of reaction to be −67,600 calories per mole.

The Effect of Alcohol.—In general denaturing agents shift the equilibrium between native and denatured trypsin towards the denatured form. 10 per cent alcohol in 0.01 N hydrochloric acid lowers the temperature of half inactivation 5.5° (*cf.* Table I).

We are now in a position to understand why trypsin is such favorable material for the study of denaturation and its reversal. The first condition for the reversal of denaturation is that the denaturation procedure used should not cause secondary irreversible changes.

TABLE II

Effect of Temperature on the Equilibrium between Native and Denatured Trypsin in 0.01 N Hydrochloric Acid

Temperature	Percentage denaturation	Percentage denaturation calculated from $\ln K = -\frac{\Delta H}{KT} + C$ $-\Delta H = 67{,}600$ calories/mole
°C.		
42	32.8	32.8
43	39.2	41.0
44	50.0	50.0
45	57.4	56.4
48	80.4	80.0
50	87.8	87.2

Trypsin is remarkably stable in acid. The second condition for reversal is that the denatured protein be brought to a pH at which the native form is the equilibrium form. In the case of some proteins this means a pH close to the isoelectric point, *i.e.* a pH at which the denatured protein is insoluble, at which it is precipitated before reversal of denaturation can take place. Denatured trypsin changes into native trypsin in acid solutions in which denatured trypsin is entirely soluble.

EXPERIMENTAL

The trypsin used was prepared according to Northrop and Kunitz (1932). For the experiments of Table I the trypsin cake was simply diluted with the solvents described. For the experiments of Table II the trypsin was first dialyzed

in the cold. Dialysis does not affect the results because so much solvent is added to the trypsin cake that the salt introduced with the cake is eventually present in extremely dilute solution.

The method of estimating active native trypsin in the presence of inactive denatured trypsin by the use of a suitable hemoglobin solution has already been described (Anson and Mirsky, 1933).

The experiments with dilute trypsin in 0.01, 0.003, and 0.001 N hydrochloric acid, which include the experiments on the effect of temperature on the percentage denaturation, were carried out as follows: To estimate the activity before heating 5 ml. of hemoglobin solution plus 0.5 ml. water were poured into 0.5 ml. enzyme solution and the digestion was carried out for 5 minutes. Exactly the same procedure was used to measure the activity after heating and cooling. The heating consisted in keeping the test-tube containing the enzyme solution in a water bath 2° above the inactivation temperature for 2 minutes. The cooling consisted in keeping the trypsin solution at 25° for 10 minutes. The enzyme solution was heated to the inactivation temperature by being kept in a water bath at the inactivation temperature for 2–3 minutes and cooled to the inactivation temperature by being kept first in a bath 1–2° above the inactivation temperature for 1–2 minutes and then in a bath at the inactivation temperature for 2–3 minutes. The time at the higher temperature sufficed to produce more than half denaturation as was determined by separate experiments.

In the case of the ten times more concentrated trypsin in 0.01 N hydrochloric acid, the enzyme was first diluted ten times and a digestion mixture of the normal composition obtained by adding to 0.5 ml. of trypsin solution 4.5 ml. of a mixture of 10 parts hemoglobin solution and 0.8 parts water and then there were added to 1 ml. of the resulting solution 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion time was measured from the first addition of hemoglobin.

In the case of the solution of trypsin in acid alcohol, the enzyme solution was kept at the inactivation temperature for 5 minutes and before being cooled to the inactivation temperature it was kept at a temperature 1.5° higher for 1 minute.

To estimate the activity in 0.05 N sodium hydroxide at 0°C. 1 ml. of 0.1 N sodium hydroxide was added to 1 ml. trypsin solution. After 1 minute there were added to 0.5 ml. of this solution a mixture of 5 ml. hemoglobin solution and 0.5 ml. 0.05 N hydrochloric acid. Digestion was carried out for 5 minutes. To find out how much of the trypsin was reversibly inactivated by the alkali, 14.5 ml. of 0.01 hydrochloric acid were added to 0.5 ml. of the alkaline trypsin and, after 10 minutes at 25°C., 5 ml. of hemoglobin solution were added to 1 ml. of the acidified trypsin and digestion was carried out for 5 minutes.

SUMMARY

There is a mobile equilibrium between the native and denatured forms of trypsin which depends on the concentrations of acid, alkali, and alcohol and on the temperature.

The heat of denaturation in 0.01 N hydrochloric acid calculated from the effect of temperature on the equilibrium constant is −67,600 calories per mole.

REFERENCES

Anson, M. L., and Mirsky, A. E., 1925, *J. Gen. Physiol.*, **9**, 169.
Anson, M. L., and Mirsky, A. E., 1933, *J. Gen. Physiol.*, **17**, 159.
Northrop, J. H., 1932, *J. Gen. Physiol.*, **16**, 323.
Northrop, J. H., and Kunitz, M., 1932, *J. Gen. Physiol.*, **16**, 267.
Kunitz, M., and Northrop, J. H., 1934, *J. Gen. Physiol.*, **17**, in press.