Photo- and Heat-Inactivation of Taka-Amylase A and Substrate Effects

Under aerobic conditions, the irradiation of taka-amylase A (TAA) solution by UV-light or, if a suitable sensitizer is present, by visible light, brings about irreversible inactivation.[1] The inactivation is mainly derived from the photo-oxidation of the constituent amino acids. On heating TAA solution[2,3], TAA undergoes certain characteristic changes in its structure, which give rise to an irreversible loss of activity. Therefore, the photoinactivation in high temperature ranges is caused by the collaboration of heat and photochemical processes. In the presence of a substrate, starch strongly protects TAA from heat- and photo-inactivation[2-4]. This phenomenon is of great interest, but the detailed mechanisms are still obscure.

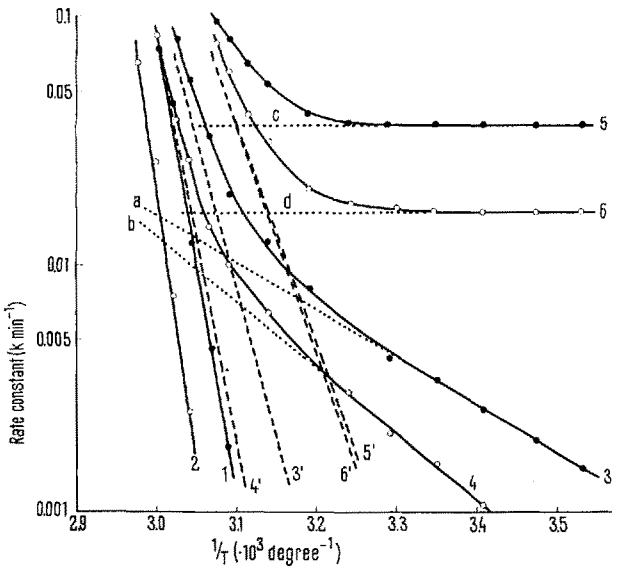
The purpose of this article is to find, by determining the thermodynamic quantities for TAA inactivations, (1) the relationship between photochemical and heat processes, and (2) to clarify the mechanism of protection by substrate.

The overall rate constants for heat, UV and visible (riboflavin as a sensitizer) inactivations, in the absence and presence of substrate, were determined from the initial velocity of inactivation. The logs of these values are plotted against the reciprocal of the absolute temperature (T) in the Figure. The plot for heat inactivation (curves 1 and 2) is exactly linear, obeying the Arrhenius law. However, the curves for photo-inactivation (curves 3, 4, 5, and 6) are not linear, consisting of low and high temperature portions. Since heat-inactivation is measurable only above about 50 °C, it is considered that the inactivation in the sufficiently low temperature range comes merely from the photochemical process. Although the exact relationship between thermal and photochemical effects in the high-temperature range may be a very complicated one, the differences (curves 3′, 4′, 5′, and 6′) between the overall rate constants (curves 3, 4, 5, and 6) and the extrapolations of the sufficiently low temperature portions (curves a, b, c, and d) were found to be linear, as seen in the Figure. Therefore, the observed overall rate constant (k) can be expressed by the addition of 2 exponential forms, the thermal and photochemical parts,

$$k = A_1 e^{-E_1/RT} + A_2 e^{-E_2/RT}$$

where A_1 and A_2 are constants, R the gas constant, and E_1 and E_2 are the activation energies for heat- and photo-inactivations respectively. However, since the heat and photochemical processes are not completely independent of each other, the first term may include the effect of the interference by the photochemical process. The activation energies calculated from curves 1 and 2, 3′, 4′, 5′, and 6′, and a, b, c, and d are given in Table I. The spontaneous breakage of the –S–S– linkage and the self-oxidation of amino acids are the main events for the UV-inactivation[1]. For this reason, the activation energy E_2 is zero. The visible inactivation[1,2] is caused by the oxidation of amino acids by the reactive O_2, which may be produced by the coupling of O_2 upon the excited sensitizer, and hence E_2 is not zero. The increase of E_2 in the presence of substrate seems to be due to the resultant effects of the increase of viscosity and the destruction of the reactive O_2 by substrate.

E_1 for UV-inactivation is much lower than that for heat-inactivation. This suggests a strong interference between heat and photochemical processes. Almost the same values of E_1 in the absence and presence of substrate, mean that the presence of substrate does not have an appreciable effect on the UV-inactivation, and the screening of the exciting light by substrate may bring about an apparent protection. In the visible inactivation, however, the smaller interference between heat and photochemical processes gives the higher value of E_1 (but



The plot of rate constants against the reciprocal of absolute temperature (T), for heat, UV and visible inactivations of taka-amylase A. Concentration of TAA, 0.005%; concentration of riboflavin, $3.3 \cdot 10^{-5} M$; pH 5.6 (the pH of the optimum activity); concentration of starch, 0.6%; exciting UV-light, light from a high-pressure mercury lamp; exciting visible light, light absorbed by 445 nm band of riboflavin. The activity was measured at 0 °C by the blue value method. ● = in the absence of starch; o = in the presence of starch; 1 and 2 = heat inactivation; 3 and 4 = visible inactivation; 5 and 6 = UV-inactivation; a, b, c, and d = the extrapolations of low temperature portions of 3, 4, 5, and 6, respectively; 3′, 4′, 5′, and 6′ = 3 – a, 4 – b, 5 – c, and 6 – d, respectively.

Table I. Activation energies for heat, UV and visible inactivations of taka-amylase A, in the absence and presence of starch

Inactivation	Activation energies (Kcal/M)	
	Thermal process E_1	Photochemical process E_2
Heat	85.2	–
	91.2 (s)	–
Ultraviolet	41.6	0
	41.8 (s)	0 (s)
Visible	57.0	8.5
	77.6 (s)	12.8 (s)

(s) = the value in the presence of substrate.

[1] S. S. KIM, Nippon Nogei Kagaku Kaishi *40*, 67, 73 (1966).
[2] G. TOMITA and S. S. KIM, Nature *205*, 46 (1965).
[3] B. HAGIHARA, T. NAKAYAMA, H. MATSUBARA, and K. OKUNUKI, J. Biochem., Tokyo *43*, 483 (1956).
[4] G. TOMITA and S. S. KIM, Experientia *22*, 27, 366 (1966).

lower than that for heat-inactivation). The formation of enzyme-substrate complex may increase the rigidness of the secondary structure of enzyme protein and may suppress the interference between heat and photochemical processes. Therefore, the presence of substrate makes E_1 higher. By applying the theory of absolute reaction rate[5,6] to curves 1, 2, 3′, 4′, 5′, and 6′, the heat of activation (ΔH^*), the free energy of activation (ΔF^*) and the entropy of activation (ΔS^*) were calculated and are given in Table II. These values decrease in the order of heat, visible and UV inactivations, depending on the degree of the interference between heat and photochemical processes. The important feature of the results in Table II is the abnormally high entropies of activation. This explains the abnormally high activation energy for the heat-inactivation. The high value of entropy of activation suggests that a profound structural change is involved. The low value of ΔS^* for the UV-inactivation is due to a strong interference between heat and photochemical processes. The values of thermodynamic quantities for the UV-inactivation are not appreciably changed by the presence of substrate. This means a lack of substrate effect, as stated above. The protective effect of substrate is thought to be attributable to the stabilization of the secondary structure of enzyme protein – the conformation change from disorder to rigid and order – by the formation of enzyme-substrate complex. Such a formation of complex gives rise to the decrease of entropy[7]. This may explain the increase of ΔS^* by the presence of substrate. Not only protection from the heat process but suppression of the interference between heat and photochemical processes by the formation of enzyme-substrate complex inhibit the visible inactivation. For this reason, the difference between ΔS^* in the absence and presence of substrate is large for the visible inactivation.

Table II. Free energies of activation (ΔF^*), heats of activation (ΔH^*) and entropies of activation (ΔS^*) for thermal processes of various inactivations

Inactivation	°C	ΔF^* (Kcal/M)	ΔH^* (Kcal/M)	ΔS^* (cal/M)
Heat	55	24.7	84.5	183
		25.9 (s)	90.5 (s)	197 (s)
Ultraviolet	50	23.7	40.9	53
		23.7 (s)	41.2 (s)	54 (s)
Visible	50	24.9	56.4	99
		25.5 (s)	77.0 (s)	160 (s)

(s) = value in the presence of substrate.

TAA forms enzyme-product complex as well as enzyme-substrate complex, so that the protections observed are considered to be due to the combined effects of substrate and its decomposition products.

Zusammenfassung. Es werden die Bestimmung der thermodynamischen Quantitäten für die Inaktivierungsreaktionen, die Wärme- und Photo-Inaktivierungen der Taka-Amylase A und die Schutzwirkung des Substrats gegen diese Inaktivierungen diskutiert.

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[5] H. EYRING, J. chem. Phys. *3*, 107 (1935).
[6] W. F. K. WYNNE-JONES and H. EYRING, J. chem. Phys. *3*, 492 (1935).
[7] The difference of 14 cal/M between the entropies for the heat-inactivation in the absence and presence of substrate (Table II) is considered to be concerned with the entropy change upon the formation of enzyme-substrate complex.

Direct Aromatization of C_{19}-Steroid Sulphates[1]

In the course of recent investigations on the in vivo biogenesis of 7α-^{3}H-dehydroepiandrosterone (DHEA) ^{35}S-sulphatide and its metabolism, estrogen sulphatides with the original ^{3}H/^{35}S ratio could be demonstrated in peripheral plasma[2]. On theoretical grounds this direct transformation of neutral C_{19}-steroid sulphatide into phenolic C_{18}-steroid sulphatide should involve the dehydrogenation of DHEA sulphatide to the corresponding dienol sulphatide of androst-4-ene-3,17-dione (androstenedione) as the first step in the reaction sequence. In order to substantiate such an hypothesis, the conversion of synthetic androstenedione sulphate into estrogens has been attempted under in vitro conditions.

The 3,5-dienol sulphate of androstenedione was prepared by routine methods[3], using chlorosulphonic acid or its methyl ester in absolute pyridine. The sodium salt of the sulphoconjugate, purified by thin layer chromatography on silica gel G in chloroform-methanol-ammonia (20:5:0.2 v/v) (Rf = 0.20), on DEAE-cellulose in isopropanol-water-formic acid (65:33:2 v/v) (Rf = 0.21) and paper chromatography on Whatman DE-20 in 1.0M acetate buffer of pH 4.7[4] (Rf = 0.36), had a melting point of 194–200 °C (uncorr.). The UV-spectrum in methanol exhibited an absorption maximum at 238 nm, the IR-spectrum strong absorption bands near 1742 cm^{-1} (17-keto group), 1638 cm^{-1} (3,5-dienol ester group), 1245 cm^{-1} and 1048 cm^{-1} (assymetric and symmetric S–O vibration), while the characteristic absorption band of Δ^4-3-keto-steroids near 1618 cm^{-1} had disappeared.

When 1.95 μg (5 nmMol) of 7α-^{3}H-androstenedione ^{35}S-sulphate-Na with 310,000 dpm ^{3}H and 161,000 dpm ^{35}S

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[2] G. W. OERTEL, P. KNAPSTEIN, and L. TREIBER, Hoppe-Seyler's Z. physiol. Chem., in press (1966).
[3] A. B. ROY, Biochem. J. *62*, 41 (1956).
[4] G. W. OERTEL and E. KAISER, Biochem. Z. *336*, 10 (1962).