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Kinetic Analysis about the Effects of Neutral Salts on the Thermal Stability of Yeast Alcohol Dehydrogenase

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The effects of salts on the rate constants of inactivation by heat of yeast alcohol dehydrogenase (YADH) at 60.0°C were measured. Different effects were observed at low and high salt concentrations. At high concentrations, some salts had stabilizing effects, while others were destabilizing. The effects of salts in the high concentration range examined can be described as follows: (decreased thermal stability) $NaClO_4$ < NaI = $(C_2H_5)_4NBr$ < NH_4Br < NaBr = KBr = CsBr = (no addition) < $(CH_3)_4NBr$ < KCl < KF < Na_2SO_4 (increased thermal stability). The decreasing effect of $NaClO_4$ on YADH controlled the thermal stability of the enzyme absolutely and was not compensated by the addition of Na_2SO_4, a salt which stabilized the enzyme. However, Na_2SO_4 compensation did occur in response to the decrease in thermal stability caused by $(C_2H_5)_4NBr$. The rate constants of inactivation by heat (k_{in}) of the enzyme were measured at various temperatures. Effective values of the thermodynamic activation parameters of thermal inactivation, activation of free energy ($\Delta G^{\ddagger}$), activation enthalpy ($\Delta H^{\ddagger}$), and activation entropy ($\Delta S^{\ddagger}$), were determined. The thermal stability of YADH in 0.8 M Na_2SO_4 increased more than that of pyruvate kinase from *Bacillus stearothermophilus*, a moderate thermophile. The changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were great and showed a general compensatory tendency, with the exception of in the case of $NaClO_4$. The temperature for the general compensation effect (T_c) was approximately 123°C. With Na_2SO_4, the thermal stability of YADH at a temperature below T_c was greater than that in the absence of salt due to the higher values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, respectively, and thus was an example of low-temperature enzymatic stabilization. With $(C_2H_5)_4NBr$, the thermal stability of YADH at a temperature below T_c was lower than that in the absence of salt due to the lower values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, respectively, and thus was an example of low-temperature enzymatic destabilization. But with $NaClO_4$, the changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were small and the thermal stability of YADH was thus an example of high-temperature enzymatic destabilization.

Key words: effects of salts on proteins, kinetics of thermal stability, salting-in, salting-out, yeast alcohol dehydrogenase.

Neutral salts at high concentrations have a profound effect on the conformational stability of biological macromolecules (*1*, *2*). The Hofmeister or lyotropic series of ions was demonstrated by Hofmeister on measurement of the relative effectiveness of ions in causing the precipitation of proteins (*3*). There are two hypotheses concerning the mechanism of interaction between enzyme proteins and concentrated salt solutions. One explanation involves the state of the water structure and its interaction with nonpolar side chains in the protein (*2*, *4–8*). An alternative explanation is that ions may interact directly with the polar groups (amides) in proteins (*9–11*). Damodaran and Kinsella (*8*) described the thermodynamics of salt–protein interactions using a protein–ligand binding system. The effects of individual ions on macromolecular stability was thought to be independent and additive (*2*). The mechanism of interaction between Hofmeister ions and peptide groups is not well understood, and it is controversial whether this interaction is ion-specific, or whether it is nonspecific and the apparent specificity resides in interactions with nearby nonpolar groups (*12*). The effects of different salts (LiCl, NaCl, ChoCl, KF, KCl, and KBr) on the structural stability of a 33-residue peptide corresponding to the leucine zipper region of GCN4 have been studied by high-sensitivity differential scanning calorimetry, and the data are consistent with the existence of specific interactions between anions and peptides exhibiting affinity that follows a reverse size order ($F^- > Cl^- > Br^-$) (*13*). Yeast alcohol dehydrogenase (YADH) contains a zinc atom in its catalytic site and the enzymatic activity of YADH is absolutely dependent on maintenance of the integrity of zinc coordination at the catalytic site (*14*).

This paper examines the effects of salts composed of monovalent cations on the rate constants of inactivation by heat at 60.0°C of yeast alcohol dehydrogenase (YADH) composed of four subunits, as well as the effects of mixing two kinds of salts, namely one that decreases and another that increases the thermal stability of YADH.

In this study, the rate constants of irreversible thermal inactivation (k_{in}) of YADH were measured under various

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conditions and at various temperatures, k_{in} being within the range of 1.1 min^{-1} to 0.03 min^{-1}, and effective values of the thermodynamic activation parameters of thermal inactivation, activation of free energy ($\Delta G^{\ddagger}$), activation enthalpy ($\Delta H^{\ddagger}$), and activation entropy ($\Delta S^{\ddagger}$), were determined by applying the absolute reaction rates equation (*15*). The general compensation effect (*16*) is simply defined as that which occurs when a series of Arrhenius plots pass through a common point. The temperature for this point has been termed the temperature of compensation (T_c). Barnes *et al.* (*17*) and Urabe *et al.* (*18*) discussed the probable biological utility of a physical constant, the aforementioned Tc. This paper looks at the changes in thermal stability of YADH in concentrated salt solutions, the changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, and the relation between thermal stability and a physical constant, T_c.

MATERIALS AND METHODS

Materials—Yeast alcohol dehydrogenase (YADH) lyophilizate and lactate dehydrogenase (LDH) from pig muscle were purchased from Roche Diagnostics. Pyruvate kinase from *Bacillus stearothermophilus* was purchased from Unitica Ltd. The enzymes purchased were homogeneous, as judged on gel electrophoresis, and thus used without further purification. The nicotinamide adenine dinucleotide (NAD), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and phosphoenol pyruvate (PEP) used were purchased from Roche Diagnostics. The NADH used was purchased from Oriental Yeast Co., Ltd. All salts used were guaranteed reagent grade and used without further purification. Glass-distilled water was used throughout.

Enzyme Activity Assay and Thermal Inactivation—The pH of the concentrated salt solutions (20 mM potassium phosphate buffer, pH 7.20 at 25.0°C) was adjusted with KOH or HCl. Solutions containing iodide were stabilized with 5 mM sodium thiosulfate. Thermal inactivation of enzymes under the various conditions was carried out at controlled temperatures. Aliquots of 1 μl were removed at certain times and immediately put into 500 μl assay mixture. The assay mixture for YADH was adjusted to pH 8.30 with KOH, and contained 2.1 mM NAD^+, 172 mM ethanol, 74.4 mM potassium pyrophosphate, 74.7 mM semicarbazide HCl and 22.2 mM glycine. The enzymatic activity of YADH was determined by measuring the increase in optical density at 340 nm on the formation of NADH. The concentration of YADH was determined by measuring the optical density at 280 nm, assuming an extinction coefficient of 1.89×10^5 cm^2 mol^{-1} based on a molecular weight of 150 kDa (*19*). The assay mixture for pyruvate kinase from *B. stearothermophilus* contained 50 mM imidazole HCl, pH 7.20, 50 mM KCl, 7 mM $MgCl_2$, 4 mM phosphoenol pyruvate (PEP), 6 mM ADP, 0.4 mM NADH, 0.1 mM AMP and 8.5 units LDH from pig muscle per 500 μl assay mixture. The enzymatic activity of pyruvate kinase from *B. stearothermophilus* was determined by measuring the decrease in optical density at 340 nm due to oxidation of NADH. The concentration of pyruvate kinase from *B. stearothermophilus* was determined by measuring the optical density at 280 nm, assuming an extinction coefficient of A_{280} = 2.31 (*20*).

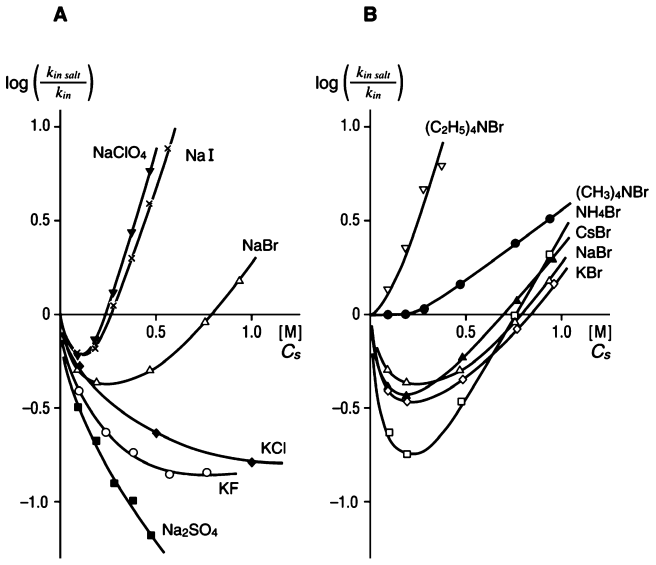

Fig. 1. **A and B: Plots of $\log(k_{in\ salt}/k_{in})$ against salt concentration (C_s).** C_s is the concentration of salt, $k_{in\ salt}$ is the rate constant of inactivation by heat at 60.0°C of yeast alcohol dehydrogenase in the presence of various salts at various concentrations, and k_{in} is the rate constant of inactivation by heat at 60.0°C of yeast alcohol dehydrogenase in the absence of salt. The enzyme solution used was 20 mM potassium phosphate buffer (pH 7.20, 25°C), and the enzyme concentration was 0.4 mg/ml.

RESULTS AND DISCUSSION

Irreversibility and First-Order Reaction Process of Inactivation by Heat of YADH—All rate constants of inactivation by heat of YADH at 60.0°C under various conditions were determined from the slope of semi-logarithmic plots of ln (relative remaining activity) *versus* the incubation time (min) for heat inactivation that gave a linear relationship. This, together with the lack of dependence of the stability on the enzyme concentration, led me to conclude that the heat inactivation of YADH was a first-order process. Accordingly, the activity loss is not due to dissociation of the subunits.

Following the classic work of Lumry and Eyring (*21*), it is usually assumed that the irreversible thermal inactivation of enzymes can be represented by the scheme

$$N \overset{K}{\rightleftarrows} D \overset{k}{\rightarrow} I$$

where N is the native, catalytically active enzyme, D is a reversibly denatured, thermally unfolded enzyme, I is an irreversibly inactivated enzyme, K is the equilibrium constant between N and D, and k is the rate constant of an irreversible component of the heat inactivation process such as incorrect structure formation or a chemical deterioration reaction. Zale and Klibanov (*22*) estimated the contribution of reversible thermal denaturation to the overall irreversible thermal inactivation process. At high temperatures at which $[N] \ll [D]$ and hence $K \ll 1$, the observed rate constant, k_{obs}, is equal to the true, monomolecular rate constant, k.

The Effects of Salts on Inactivation by Heat of YADH at 60.0°C—The effects of salts composed of monovalent cations on the rate constants of inactivation by heat of

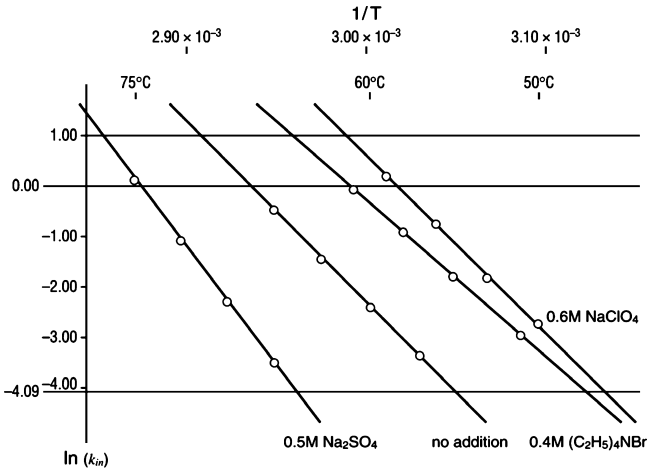

Fig. 2. **Arrhenius plot of ln(k_{in}) relative to 1/*T*.** Arrhenius plots for the heat inactivation rate constant (k_{in}) of yeast alcohol dehydrogenase in the presence and absence of a salt, with lines drawn at ln(k_{in}) = 0.00 and ln(k_{in}) = –4.09. k_{in} is in min^{-1}.

YADH at 60.0°C were examined. The salts examined were $NaClO_4$, NaI, NaBr, CsBr, KBr, KCl, KF, Na_2SO_4, NH_4Br, $(CH_3)_4NBr$ and $(C_2H_5)_4NBr$. The relationship of log ($k_{in\ salt}/k_{in}$) to the concentration of salt (C_s) is shown in Fig. 1, where $k_{in\ salt}$ is the rate constant of inactivation by heat at 60.0°C in the presence of a salt and k_{in} is the rate constant of inactivation by heat at 60.0°C in the absence of the salt. With all the salts tested, except alkylammonium bromide, increased heat stability was found at concentrations lower than approximately 0.2 M. At near physiological concentrations (1–200 mM), a salt can have a significant impact on the stability of the structure of macromolecules such as a highly charged nucleic acid structure and a more weakly charged protein structure (*23*). The common effect of electrostatic interaction of ions with the charge of protein molecules might play a role in enzymatic stabilization. Ammonium bromide showed the strongest effect as to this protection, but neither $(CH_3)_4NBr$ nor $(C_2H_5)_4NBr$ showed this protective effect at a low concentration.

The Hofmeister effect, which occurs at still higher salt concentrations, may strengthen the hydrophobic force by increasing the surface tension of the solvent, or stabilize peptide dipoles through specific ionic interactions (*12*, *23*, *24*). When the concentrations of salts were high, each salt had a different effect on the thermal stability of YADH. The effects of salts in the high concentration range examined can be described as follows: (decreased thermal stability) $NaClO_4$ < NaI = $(C_2H_5)_4NBr$ < NH_4Br < NaBr = KBr = CsBr = (no addition) < $(CH_3)_4NBr$ < KCl < KF < Na_2SO_4 (increased thermal stability). $NaClO_4$, the salt which increased the rate constant of inactivation most, and Na_2SO_4, the salt which stabilized the enzyme most, did not compensate for each other's effect, as shown in Table 1. On the other hand, in a mixture of 0.5 M $(C_2H_5)_4NBr$ and 0.5 M Na_2SO_4, the rate constant nearly equaled the value found in the absence of the salts. The effect of $(C_2H_5)_4NBr$, which decreased the thermal stability of YADH, was compensated by the addition of Na_2SO_4, the thermal stability of YADH increasing. Therefore, the

Table 1. **Rate constants of irreversible heat inactivation of yeast alcohol dehydrogenase (k_{in}) at 60.0°C under various conditionsa.**

	k_{in} (min^{-1}) at 60.0°C
No Addition	0.0891 (1)
+ 0.5 M $NaClO_4$	0.514 (×5.8)
+ 0.5 M $(C_2H_5)_4NBr$	1.16 (×13.0)
+ 0.5 M Na_2SO_4	0.00661 (×0.074)
+ 0.5 M $NaClO_4$ + 0.5 M Na_2SO_4	0.537 (×6.0)
+ 0.5 M $(C_2H_5)_4NBr$ + 0.5 M Na_2SO_4	0.128 (×1.4)

[a] The enzyme solution used was 20 mM potassium phosphate buffer (pH 7.20, 25°C), and the enzyme concentration was 0.4 mg/ml.

Table 2. **K_s' for yeast alcohol dehydrogenase using rate constants of irreversible heat inactivation at 60.0°C, and K_s for acetyltetraglycine ethyl ester (ATGEE) using solubility measurements.[a]**

	K_s' for yeast ADH	K_s for ATGGEE*
$NaClO_4$	–3.51 (×11)	–0.33 (1)
NaI	–3.23 (×14)	–0.23 (1)
$(C_2H_5)_4NBr$	–3.10 (×28)	–0.11 (1)
NH_4Br	–1.76 (×16)	–0.11 (1)
KBr	–1.25	–0.023
CsBr	–1.18	—
NaBr	–1.16	0.00
$(CH_3)_4NBr$	–0.69	+0.018
KCl	+0.57	+0.046
KF	+1.09 (×5)	+0.23 (1)
Na_2SO_4	+2.14 (×5)	+0.48 (1)

[a] K_s' is the slope of the linear part of Fig. 1. K_s for ATGEE was determined by Robinson and Jencks (*9*).

mechanisms of thermal stability decrease for YADH caused by $(C_2H_5)_4NBr$ and $NaClO_4$ were not identical.

The Effects of Anions and Cations on Thermal Stability and Salting-Out Constants of YADH—In order to simplify the conditions, monovalent cations were used throughout this study. As nearly the same effect was observed when the bromide salts of Na^+, K^+ and Cs^+ were used (Fig. 1B), alkali metal ions might have a similar effect on heat inactivation of the enzyme. Na^+ and K^+ were used to study the sensitivity to the nature of the anion, as shown in Fig. 1A.

The plots in Fig. 1A exhibited straight lines when the salt concentration was high. Because Na^+ and K^+ showed the same effect, the slope of this plot depends on the nature of the anion. Each halide ion had a different effect on the rate constant of heat inactivation, while there was a tendency towards a decrease in thermal stability with an increase in the size of the anion. A similarly high sensitivity to the nature of the anion, which generally follows the Hofmeister series, was found with the other salts examined. ClO_4^- is a large anion and decreases thermal stability as much as I^-. SO_4^{2-}, which is a divalent anion, increased thermal stability more than F^-, which is the smallest anion.

The effects of salts on the solubility of proteins generally follow the Setschenow equation with a logarithmic relationship between solubility and salt concentration: log (S/S_0) = $-K_s \cdot C_s$, where S_0 and S are the molar solubilities of proteins in water and in a salt solution, respectively, K_s is the salting-out constant, and C_s is the molar

Table 3. **Changes in thermal stability of enzymes due to the effects of salts and the activation of free energy of heat inactivation ($\Delta G^{\ddagger}$).**[a]

A. Temperature (T_0) at which $\ln(k_{in})$ is equal to 0.00, namely (v/v_0) changes from 1 to 1/*e* in 1 min, with activation of free energy ($\Delta G^{\ddagger}$).

	T_0 (°C)	ΔT_0 °C)	$\Delta G^{\ddagger}$(kcal·mol^{-1})	$\Delta(\Delta G^{\ddagger})$ (kcal·mol^{-1})
YADH				
+ 0.6 M $NaClO_4$	58.5	–9.1	22.17	–0.62 (–2.7%)
+ 0.6 M $(C_2H_5)_4NBr$	58.3	–9.3	22.15	–0.64 (–2.8%)
+ 0.5 M $(C_2H_5)_4NBr$	59.6	–7.9	22.25	–0.54 (–2.4%)
+ 0.4 M $(C_2H_5)_4NBr$	61.3	–6.3	22.36	–0.43 (–1.9%)
no addition	67.6	—	22.79	—
+ 0.5 M Na_2SO_4	74.8	+7.2	23.29	+0.50 (+2.2%)
+ 0.8 M Na_2SO_4	77.4	+9.9	23.47	+0.68 (+3.0%)
PK from *B. stearo*				
no addition	75.7	+8.1	23.35	+0.56 (+2.5%)

B. Temperature (T_{-4}) at which $\ln(k_{in})$ is equal to –4.09, namely (v/v_0) changes from 1 to 1/*e* in 60 min, with activation free energy ($\Delta G^{\ddagger}$).

	T_{-4} (°C)	ΔT_{-4} (°C)	$\Delta G^{\ddagger}$ (kcal·mol^{-1})	$\Delta(\Delta G^{\ddagger})$ (kcal·mol^{-1})
YADH				
+ 0.6 M $NaClO_4$	45.9	–8.7	23.89	–0.68 (–2.8%)
+ 0.6 M $(C_2H_5)_4NBr$	43.4	–11.3	23.70	–0.87 (–3.5%)
+ 0.5 M $(C_2H_5)_4NBr$	45.2	–9.4	23.84	–0.73 (–3.0%)
+0.4 M $(C_2H_5)_4NBr$	47.2	–7.5	23.99	–0.58 (–2.4%)
no addition	54.6	—	24.57	—
+ 0.5 M Na_2SO_4	64.5	+9.8	25.32	+0.75 (+3.1%)
+ 0.8 M Na_2SO_4	67.7	+13.0	25.57	+1.00 (+4.1%)
PK from *B. stearo*				
no addition	65.4	+10.7	25.39	+0.82 (+3.3%)

[a] The activation of free energy of heat inactivation ($\Delta G^{\ddagger}$) was calculated with the absolute reaction rate equation (*15*), $\Delta G^{\ddagger} = RT[\ln(k_B T/h) - \ln(k_{in})]$, where R is the gas constant, T is the absolute temperature, k_B is Boltsman's constant, and h is Plank's constant. YADH is yeast alcohol dehydrogenase and PK from *B. stearo* is pyruvate kinase from *B. stearothermophilus*. v/v_0 is relative remaining activity.

concentration of the salt. Robinson and Jencks (*9*) determined K_s of an uncharged model peptide, acetyltetraglycine ethyl ester (ATGEE), in the presence of a concentrated salt solution by means of solubility measurements. The relation between $\log(k_{in\ salt}/k_{in})$ and C_s shows a linear relationship with a high salt concentration (Fig. 1), and is expressed by the following equation:

$$\log(k_{in\ salt}/k_{in}) = -K_s' \cdot C_s + \alpha$$

where K_s' is the slope of the linear part of dependence. It is impossible to determine K_s of a protein in the presence of salting-in salts by solubility measurement because the protein is denatured before equilibrium is achieved. Table 2 shows a comparison between K_s' for YADH determined from rate constants of heat inactivation and K_s for ATGEE determined by solubility measurements. The dependence of K_s' for YADH closely matches the order of K_s for ATGEE with the exception of $(C_2H_5)_4NBr$. This exception shows a difference between a real protein and the model peptide ATGEE. The ethyl group of $(C_2H_5)_4N^+$ destabilizes the hydrophobic bonds that are largely buried in the interior of the real protein. Of note is that K_s' for YADH with KF and with Na_2SO_4 are only about five times larger than K_s for ATGEE. This suggests that the increase in thermal stability of YADH could be accounted for if the equivalent of approximately five ATGEE units were removed from exposure to the solvent at the time of the increase in thermal stability. Additionally, K_s' for YADH with $NaClO_4$, NaI and NH_4Br are negative and from about 11 to 16 times smaller than K_s for ATGEE. This suggests that the decrease in the thermal stability in YADH could be accounted for if the equivalent of approximately 11 to 16 ATGEE units were exposed to the solvent at the time of the decrease in thermal stability.

Kinetic Analysis of the Effects of Neutral Salts on the Thermal Stability of the Enzyme Protein—This analysis was performed in accordance with the method of Ikegaya *et al.* (*25*). All rate constants of inactivation by heat (k_{in}) of enzymes under various conditions were determined from the slope of semi-logarithmic plots of ln (relative remaining activity) *versus* incubation time (min) for heat inactivation. The temperature for heat inactivation was set so that k_{in} would be within the range of 1.1 to 0.03 min^{-1}. The Arrhenius plots for the measured rate constant (k_{in}) of thermal inactivation under all conditions in this study were linear.

Figure 2 shows the Arrhenius plots for heat inactivation rate constants (k_{in}) of YADH under various conditions. In this study, k_{in} is in min^{-1}. T_0 is the temperature at the intersecting point of each Arrhenius plot with cross axis $\ln(k_{in}) = 0.00$, at which the remaining relative activity changes from 1 to 1/*e* in 1 min. T_{-4} is the temperature at the intersecting point of each Arrhenius plot with cross axis $\ln(k_{in}) = -4.09$, at which the remaining relative activity changes from 1 to 1/*e* in 60 min. Changes in thermal stability were analyzed quantitatively by means of comparison of T_0 and T_{-4}, respectively, under various conditions.

The values of $\Delta G^{\ddagger}$ at T_0 and T_{-4} were calculated with the absolute reaction rate equation (*15*), and are listed in

Table 4. **Activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of heat inactivation of enzymes.**[a]

	$\Delta H^{\ddagger}$ (kcal·mol^{-1})	$\Delta(\Delta H^{\ddagger})$ (kcal·mol^{-1})	$\Delta S^{\ddagger}$ (cal·mol^{-1}·deg^{-1})	$\Delta(\Delta S^{\ddagger})$ (cal·mol^{-1}·deg^{-1})
YADH				
+ 0.6 M $NaClO_4$	68.1	–2.0 (–2.9%)	140	+1.4 (+1.0%)
+ 0.6 M $(C_2H_5)_4NBr$	56.6	–13.5 (–19.3%)	107	–30.7 (–22.2%)
+ 0.5 M $(C_2H_5)_4NBr$	59.4	–10.7 (–15.3%)	114	–24.3 (–17.6%)
+ 0.4 M $(C_2H_5)_4NBr$	61.2	–8.9 (–12.7%)	117	–21.4 (–15.5%)
no addition	70.1	—	138	—
+ 0.5 M Na_2SO_4	92.2	+22.1 (+31.5%)	200	+61.9 (+44.8%)
+ 0.8 M Na_2SO_4	98.8	+28.7 (+40.9%)	210	+72.0 (+52.1%)
PK from *B. stearo*				
no addition	92.2	+22.1 (+31.5%)	207	+68.7 (+49.7%)

[a] $\Delta H^{\ddagger}$ was calculated from the slope $[-(T + \Delta H^{\ddagger}/R)]$ of the Arrhenius plot of $\ln(k_{in})$ relative to $1/T$, and $\Delta S^{\ddagger}$ was calculated from the slope $[\ln(k_B/h) + \Delta S^{\ddagger}/R]$ of the plot of $T{\cdot}\ln(k_{in}/T)$ relative to T. R is the gas constant, T is the absolute temperature, k_B is Boltsman's constant, and h is Plank's constant. YADH is yeast alcohol dehydrogenase and PK from *B. stearo* is pyruvate kinase from *B. stearothermophilus*.

Table 3. The thermal stability of pyruvate kinase from *B. stearothermophilus*, a moderate thermophile, was compared with that of YADH in the absence of salt. The value of T_0 of pyruvate kinase increased to 75.7°C (+8.1°C), and the value of $\Delta G^{\ddagger}$ increased to 23.35 kcal·mol^{-1} (+2.5%). The thermal stability of YADH with 0.8 M Na_2SO_4 increased, while the value of T_0 was higher than that of pyruvate kinase by 1.7°C. The value of T_{-4} of pyruvate kinase increased to 65.4°C (+10.7°C) and the value of $\Delta G^{\ddagger}$ increased to 25.39 kcal·mol^{-1} (+3.3%). The thermal stability of YADH with 0.8 M Na_2SO_4 increased, while the value of T_{-4} was higher than that of pyruvate kinase by 2.3°C.

Activation enthalpy for the heat inactivation of enzymes ($\Delta H^{\ddagger}$), as derived from the slope of the Arrhenius plot relating $\ln(k_{in})$ with $1/T$, and the activation entropy for the heat inactivation of enzymes ($\Delta S^{\ddagger}$) are listed in Table 4. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of pyruvate kinase were compared, respectively, with those of YADH in the absence of salt. The value of $\Delta H^{\ddagger}$ of pyruvate kinase increased to 92.2 kcal·mol^{-1} (+31.5%) and that of $\Delta S^{\ddagger}$ increased to 207 cal·mol^{-1}·deg^{-1} (+49.7%). With the exception of $NaClO_4$, the changes in the values of both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were large and showed a general tendency toward compensation.

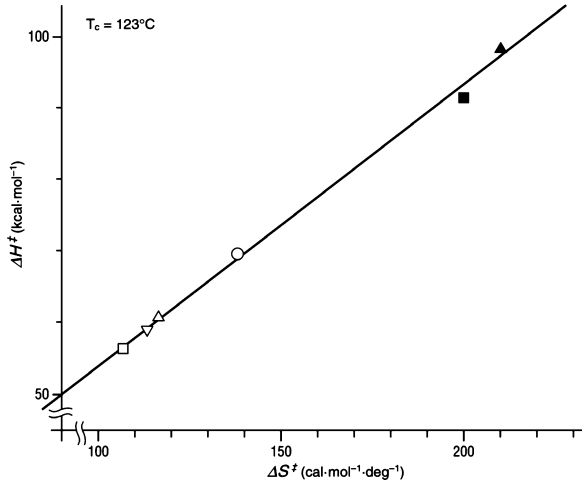

Fig. 3. **Linear relationship between activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of heat inactivation of yeast alcohol dehydrogenase in Table 4.** The conditions were no addition (open circles), 0.4 M $(C_2H_5)_4NBr$ (open triangles), 0.5 M $(C_2H_5)_4NBr$ (inverted open triangles), 0.6 M $(C_2H_5)_4NBr$ (open squares), 0.5M Na_2SO_4 (solid squares), and 0.8M Na_2SO_4 (solid triangles).

Systems exhibiting a general compensation effect show linear variation in the changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, while the mechanism of the reaction and the nature of the transition state are identical. Calculations were performed for the value of T_c using the slope in Fig. 3 and relating the value of $\Delta H^{\ddagger}$ with that of $\Delta S^{\ddagger}$. The plots in Fig. 3 show good linearity, with a slope of about 123°C (T_c). However, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ with $NaClO_4$ were excluded. With 0.8 M Na_2SO_4, the thermal stability of YADH at a temperature below the temperature of compensation (123°C) was greater than that in the absence of salt due to the higher values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Table 4). Moreover, the values of T_0 and T_{-4} were higher than those in the absence of salt by 9.9°C and 13.0°C, respectively (Table 3). Thus, the increase in thermal stability in comparison with at T_{-4} was greater than that in comparison with at T_0, providing an example of low-temperature enzymatic stabilization. With 0.6 M $(C_2H_5)_4NBr$, the thermal stability of yeast YADH at a temperature below the temperature of compensation (123°C) was lower than that in the absence of salt due to the lower values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Table 4). Further, the values of T_0 and T_{-4} were lower than those in the absence of salt by 9.3°C and 11.3°C, respectively (Table 3). The decrease, therefore, in thermal stability in comparison with at T_{-4} was greater than that in comparison with at T_0, and this is an example of low-temperature enzymatic destabilization. Although with 0.6 M $NaClO_4$ the thermal stability of YADH was less than that in the absence of salt (Table 3), the changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were quite small (Table 4). Because the values of T_0 and T_{-4} were lower than those in the absence of salt by 9.1°C and 8.7°C, respectively (Table 3), this is an example of high-temperature enzymatic destabilization.

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