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# The Biochemical Characterization of D-Hydroxyisovalerate Dehydrogenase, a Key Enzyme in the Biosynthesis of Enniatins

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**Abstract:** The biochemical properties of purified D-hydroxyisovalerate dehydrogenase from *Fusarium sambucinum* was elucidated. D-Hydroxyisovalerate dehydrogenase produced solely D-hydroxyisovalerate from 2-ketoisovalerate. The isoelectric point of the purified enzyme was 7.0. The enzyme was highly specific with 2-ketoisovalerate ($K_m$=0.188 mM, $V_{max}$=8.814 mmol/min·mg) and 2-keto-3-methyl-n-valerate ($K_m$=0.4 mM, $V_{max}$=1.851 mmol/min·mg) for the reductive reaction. This was also seen by comparing D-hydroxyisovalerate ($K_m$=1.667 mM, $V_{max}$=0.407 mmol/min·mg) and D-hydroxy-3-methyl-n-valerate ($K_m$=6.7 mM, $V_{max}$=0.648 mmol/min·mg) for the oxidative reaction. Thiol blocking reagents, such as iodoacetamide, N-ethylmaleimide and *p*-chloromecuribenzoate inhibited about 80% of enzyme activity at 0.02 mM, 50 mM and 50 mM, respectively. The enzyme activity was also inhibited by the addition of 0.1 mM of various metal ions, such as $Fe^{2+}$ (67%), $Cu^{2+}$ (88%), $Zn^{2+}$ (76%) and $Mg^{2+}$ (9%). The enzyme was stable over three months in 50 mM potassium phosphate buffer (pH 5~7) at −80℃. However the purified enzyme lost 30% of its activity in the same buffer after 24 h at 4℃. The studies about thermal inactivation of D-hydroxyisovalerate dehydrogenase exhibit 209.2 kJ/M of activation enthalpy and 0.35 kJ/mol·K of activation entropy.
**Key words;** D-hydroxyisovalerate dehydrogenase, enniatin, *Fusarium sambucinum*.

**D**-Hydroxyisovalerate dehydrogenase (D-HIV dehydrogenase) has an important role in the biosynthesis of some depsipeptides and peptolides, such as enniatins (Audhya *et al.*, 1973), beauvericin (Peeters *et al.*, 1983), destruxin (Pais *et al.*, 1981), valinomycin (Smith *et al.*, 1975), bassinolide (Okumura, 1983) and cyclosporin like peptolide (Dryfuss *et al.*, 1988), all of which contain D-hydroxyisovalerate (D-HIV) (Table 1). Enniatins are produced by several strains of the genus *Fusarium* and exhibit antibiotic properties due to their ionophoric activity. Enniatins are synthesized by a multifunctional enzyme called enniatin synthetase from their primary precursors D-HIV and a branched-chain amino acid with the consumption of S-adenosyl-L-methionine and ATP (Zocher *et al.*, 1976, 1982, 1983; Billich and Zocher, 1987). D-HIV is an important intermediate in the biosynthetic pathway of enniatins and is produced by D-HIV dehydrogenase which catalyzes the reversible reduction of 2-ketoisovalerate (2-KIV) to D-HIV in the presence of NADPH (Lee *et al.*, 1992) in *Fusarium*. Although D-HIV dehydrogenase of *F. sambucinum* was purified and characterized in our previous work (Lee *et al.*, 1992), many of its biological properties are still unknown and the function of D-HIV dehydrogenase related to the biosynthetic mechanism of enniatin has been unexplored. In this paper we described the biological activities of D-HIV dehydrogenase and related information about the role of the enzyme in the enniatin biosynthesis.

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## Materials and Methods

### Materials

Chemicals were of the highest purity commercially available. 2-KIV was purchased from Sigma (St. Louis, USA). D-HIV was a product of Serva (Heidelberg, Germany). $NADP^+$ and NADPH were obtained from Boehringer (Mannheim, FRG). Mono-Q and S-Sepharose Fast Flow were from Pharmacia (Uppsala, Sweden).

### Enzyme preparation

The maintenance and the cultures of *F. sambucinum* were done using the methods of Madry *et al.* (1983).

**Table 1.** D-HIV containing peptide antibiotics

| Name | Structure type | Structure | Organism |
|---|---|---|---|
| Beauvericin | C-depsi-6-M | ($_{NMe}$Phe-D-HIV)$_3$ | *Beauveria bassiana* |
| Bassinolide | C-depsi-8-M | ($_{NMe}$Leu-D-HIV)$_4$ | *Beauveria bassiana* |
| Destruxin | C-6-M | Pro-Ile-$_{NMe}$Val<br>\| \|<br>D-HIV-β-Ala-$_{NMe}$Val | *Metarrhizium anisopliae* |
| Enniatin | C-depsi-6-M | ($_{NMe}$Val-D-HIV)$_3$ | *Fusarium scirpi* |
| Peptolide | C-11-M | $_{NMe}$Leu-Leu-$_{NMe}$Val-$_{Me}$Bmt-Thr<br>\| \|<br>D-HIV-Ala-$_{NMe}$Leu-Leu-$_{NMe}$Leu-Sar | *Cylindrotrichum oligospermum* |
| Valinomycin | C-depsi-12 | (L-Lac-Val-D-HIV-D-Val)$_3$ | *Streptomyces tsusimaensis* |

C : cyclo; depsi : depsipeptide; M : modified; MeBmt : (2S, 3R, 4R, 6E)-methylamino-3-hydroxy-4-methyl-6-octanic acid; Sar : sarcosine.

The enzyme used in our studies was prepared by a previous procedure (Lee *et al.*, 1992).

**Protein determination**

Protein concentrations were determined by a modified Bradford procedure (1976) with bovine serum albumin as a standard.

**SDS-PAGE**

SDS-PAGE was done as described by Laemmli (1970). The gel contains 10% acrylamide and 0.2% bisacrylamide and it was silver stained using the procedure described by Blum *et al.* (1987).

***Measurement of enzyme activity***

The reactions were carried out by a previous procedure (Lee *et al.*, 1992). For the assay of the reverse reaction, the mixture contained 50 mM Tris-HCl buffer (pH 8.9), 2.85 mM $NADP^+$, 5.7 mM D-HIV, and enzyme in a final volume of 0.35 ml. The increase in the rate of reduction of $NADP^+$ due to oxidation of D-HIV was measured at 340 nm (45℃).

**Isoelectric focusing**

The isoelectric point of the enzyme was determined by using Servalyt Precoates (Serva, Heidelberg, Germany) according to the manufacturer's instruction. After 0.2 ml of Bayol F was distributed over the cooling plate of electrophoresis apparatus (Ultrophor, LKB) as heat exchange liquid, the precooled precoate (4℃) was rolled onto the cooling plate to avoid air bubbles. After peeling off the cover sheet, each wick was saturated with anode fluid (3.3 g of L-aspartic acid and 3.7 g of L-glutamic acid in 1 L of distilled water, pH 3) and cathode fluid (4 g of L-lysine and 120 ml of ethylenediamine in 1 L of distilled water, pH 10). The purified enzyme (1 μg) and Serva protein test mixture were directly loaded onto pre-cooled isoelectric focusing gel (125×125 mm), pH 3~10. Electrophoresis was carried out with voltage gradient from 0.2 kV to 2 kV (4 watt). After focusing, the gel was fixed with solution of 36% methanol, 6% trichloroacetic acid, 3.6% sulfosalycilic acid, and was stained with Coomassie Brilliant Blue W. The relative isoelectric point of the enzyme was determined from its mobility relative to those of Serva protein test mixture.

**Determination of D-HIV produced by D-HIV dehydrogenase**

D-HIV produced by the enzyme was identified by using Chiralplate (Macherey-Nagel, Germany) with authentic D-HIV and L-HIV as a standard (Guenter, 1988). The reaction mixture contained 3 U of D-HIV dehydrogenase, 10 μl of NADPH (20 mM), 10 μl of 2-KIV (50 mM) and 240 μl of potassium phosphate buffer (50 mM, pH 7.0). After incubation for 1 h at 37℃, the reaction was stopped by the decrease of pH to 2.0 with 1 N HCl. Protonated D-HIV was extracted with 2 ml ethylacetate from the reaction mixture. After concentrating D-HIV with a rotary evaporator, it was chromatographed on Chiralplate at room temperature (dichloromethane : methanol=45 : 5 (v/v)). For the staining of D-HIV, 1.82 g of vanadium pentoxide (Merck, Art. 824) was weighed into a 100 ml measuring flask, 30 ml of 1 M sodium carbonate was added and completely dissolved by treatment in an ultrasonic bath. After cooling, 46 ml of 2.5 M sulfuric acid and 23 ml of acetonitrile were added to the solution. The dried plate was dipped into this solution for 2 sec and then left to stand at room temperature until blue color of hy-

droxy acids formed on a yellow background.

### Stability of D-HIV dehydrogenase

For the investigation of thermal stability, the enzyme (1.7 U) in 50 μl of 50 mM potassium phosphate buffer (pH 7.0) was incubated at various temperatures. After shortly incubation (1 min) of the enzyme solution at 35℃ to return to the reaction temperature, the enzyme solution was added to the reaction buffer (300 μl of 50 mM potassium phosphate buffer (pH 7.0, 35℃) containing 0.29 mM of NADPH and 7 mM of 2-KIV). The residual activity was measured and expressed as a percentage of the control. The kinetics of thermal inactivation of D-HIV dehydrogenase was performed by the incubation of the enzyme up to 40 min at 35℃, 40℃, 45℃, and 50℃. The residual activity of the enzyme was measured and expressed as a percentage of the control as described above.

pH-stability of D-HIV dehydrogenase was examined in following three buffer: 50 mM potassium phosphate buffer (pH 5~8), 50 mM Tris-HCl buffer (pH 7~11), and 50 mM sodium acetate buffer (pH 4~5.3). The enzyme (1.7 U) in 50 μl of each buffer was incubated for 1 h at 25℃, for 1 day at 4℃ and for three months at −80℃ and the residual activity was measured.

## Results and Discussion

### Purification of D-HIV dehydrogenase

So far, D-HIV dehydrogenase has been purified and partially characterized in *F. sambucinum* (Lee *et al.*, 1992). However due to the instability and poor recovery of purified enzyme, many of its biochemical properties are still unknown. In order to obtain enough active purified D-HIV dehydrogenase, repeated mutation of *F. sambucinum* with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was performed for the isolation of high enniatin producing species according to Madry *et al.* (1983). This made it possible to find a mutant containing a large amount of D-HIV dehydrogenase and the mutant was used for all experiments. The rapid NaCl gradient (0~1 M) on S-sepharose Fast Flow HR 5/5 cation exchange FPLC chromatography (Pharmacia) was used for the final concentration of D-HIV dehydrogenase instead of lyophilization. As shown in Fig. 1, purified enzyme (1 μg) exhibits a single band in SDS-PAGE after silver staining (lane A and B). In our previous paper (Lee *et al.*, 1992), we showed that the enzyme consists of a single polypeptide chain with a molecular mass of about 53 kDa. With this purified enzyme further experiments were performed.

### Isoelectric focusing

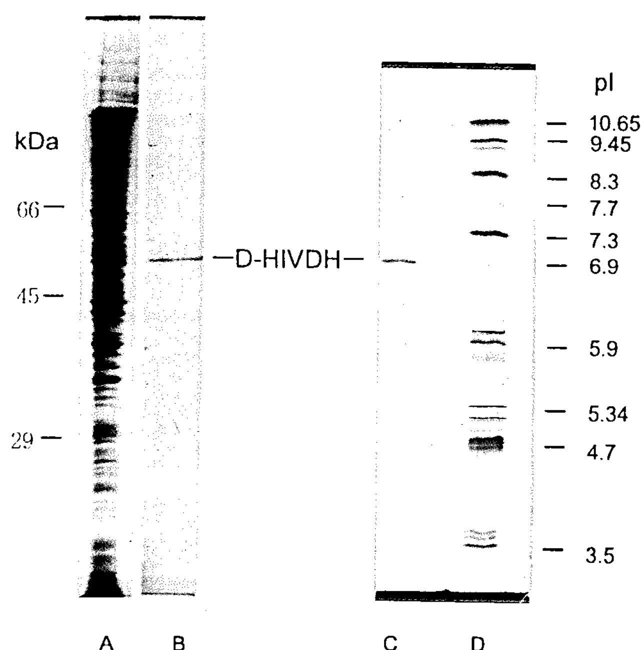


**Fig. 1.** SDS-PAGE of purified D-HIV dehydrogenase and determination of its isoelectric point. Lane A: 35~45% saturated $(NH_4)_2SO_4$ precipitation (50 μg); Lane B: purified D-HIV dehydrogenase of S-Sepharose cation exchange chromatography (1 μg); Lane C: isoelectric focusing of purified D-HIV dehydrogenase (1 μg); Lane D: Serva protein test mixture. D-HIVDH: D-HIV dehydrogenase

After purification of D-HIV dehydrogenase, isoelectric point of the enzyme was determined by using Servalyt Precoates (Serva, Heidelberg, Germany). Serva protein test mixture was used to compare isoelectric point of D-HIV dehydrogenase. From the standard curve of test proteins we could estimate the isoelectric point of D-HIV dehydrogenase as 7.0 (Fig. 1, lane C and D).

### Identification of reaction product

Thin layer chromatography using Chiralplate based on ligand exchange has been used for control of optical purity. With this system, the separation of the enantiomers is achieved without derivatization by means of ligand exchange on a reversed-phase silica gel as stationary phase which is covered with a chiral selector (proline derivative).

After discovering the new enzyme D-HIV dehydrogenase in cell of *F. sambucinum*, we carried out screening of the enzyme products. Because of the higher sensitivity of the enzyme we selected an assay procedure based on the reduction of 2-KIV as substrate. The reduction of 2-KIV can lead the formation of D-HIV and L-HIV. The stereospecificity of the enzyme product was determined on Chiralplate (Macherey-Nagel, Germany) with authentic D-HIV and L-HIV as a standard (Guenter, 1988). After the separation of reac-

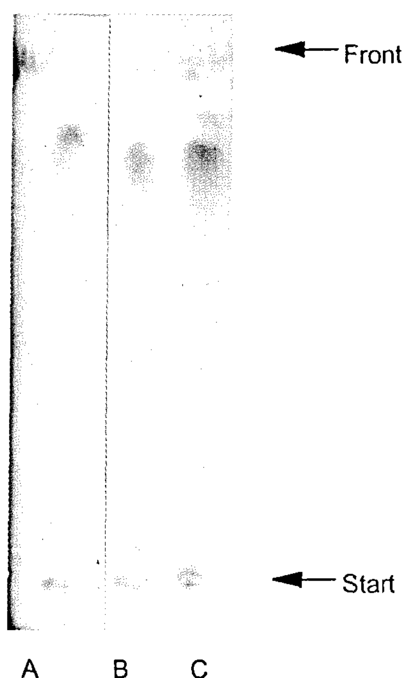


**Fig. 2.** Product identification of D-HIV dehydrogenase by thin layer chromatography using Chiralplate based on ligand exchange. Lane A: L-HIV; lane B: D-HIV; lane C: reaction products of D-HIV dehydrogenase.

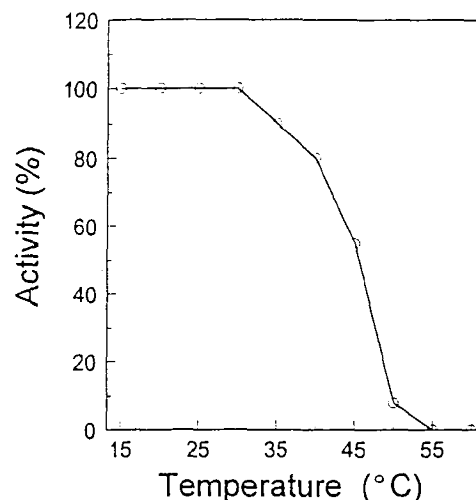


**Fig. 3.** Effect of temperature on the activity of enzyme. For the investigation of thermal stability the mixtures were preincubated at various temperatures for 10 min. The residual activities of the enzyme were measured in 50 mM potassium phosphate buffer (pH 7.0, 35℃) containing 0.29 mM of NADPH and 7 mM of 2-KIV and expressed as a percentage of the control.

tion products by TLC on Chiralplate, spots could be detected with Rf-value of 0.81 and 0.87 (Fig. 2). The Rf-values for D-HIV and L-HIV were 0.81 and 0.83, respectively. The spot from the reaction products with Rf-value of 0.87 was identified as unreacted 2-KIV. These results indicate that D-HIV dehydrogenase produces solely D-HIV from 2-KIV.

**Kinetic parameter determination**

NAD(P)H-dependent carbonyl oxidoreductases catalyze the reduction of keto carboxylic acids (Meister, 1950; Hummel *et al.*, 1983, 1985; Schuettte *et al.*, 1984). Under standard assay conditions 2-keto- or D-hydroxy acid were replaced with 2-keto-acid analogues for reductive reaction or D-hydroxy acid analogues for oxidative reaction which were close in structure to the natural substrate 2-KIV and D-HIV, respectively. The results are shown in Table 2. As previously described (Lee *et al.*, 1992), D-HIV dehydrogenase was very specific for 2-KIV ($K_m$=0.188 mM), rather than 2-keto-3-methyl-n-valerate ($K_m$=0.40 mM), which exhibited a $K_m$ value about 2 times higher than for 2-KIV. In the same way, the $V_{max}$ of D-HIV dehydrogenase toward 2-KIV (8.814 mmol/min·ml) is higher than that of 2-keto-3-methyl-n-valerate (1.851 mmol/min·ml). These findings indicate that the branching $CH_3$-group in β-position was essential for the substrate to enter the binding site of the enzyme. This was also seen by comparing D-HIV and D-hydroxy-3-methyl-n-valerate for the oxidative reaction. Among the various D-hydroxy acids, only D-HIV ($K_m$=1.667 mM, $V_{max}$=0.407 mmol/min·ml) and D-hydroxy-3-methyl-valerate ($K_m$=6.7 mM, $V_{max}$= 0.648 mmol/min·ml) were acceptable to the enzyme.

The information about substrate specificity and related maximal velocity of purified D-HIV dehydrogenase were important because of the possibility of producing D-HIV analogues by the enzyme and of incorporation into the structure of peptide antibiotics. In the biosynthetic pathway of peptide antibiotics in cells, many multi functional synthetases exhibit low substrate specificity towards amino acids and hydroxy acids yielding many product analogues (Lawen and Zocher, 1990; Pieper *et al.*, 1992). Therefore, it is possible that analogues of D-HIV produced by D-HIV dehydrogenase in cells can be incorporated into the structure of D-HIV containing peptide antibiotics by synthetases and thus new products can be formed.

**Dependence on reaction temperature**

The enzyme activity was affected by various factors such as the concentration of substrates, cofactors, pH, ionic strength and temperature. As previously described (Lee *et al.*, 1992) the optimal temperature of D-HIV dehydrogenase reaction was determined as 35℃ for the reductive reaction and 45℃ for the oxidative reaction in Tris-HCl buffer (pH 8.9). The enzyme activity for the reduction was very unstable at 45℃, losing half of its activity in 10 min (Fig. 3). These results indicated that the enzyme activity might be stabilized by

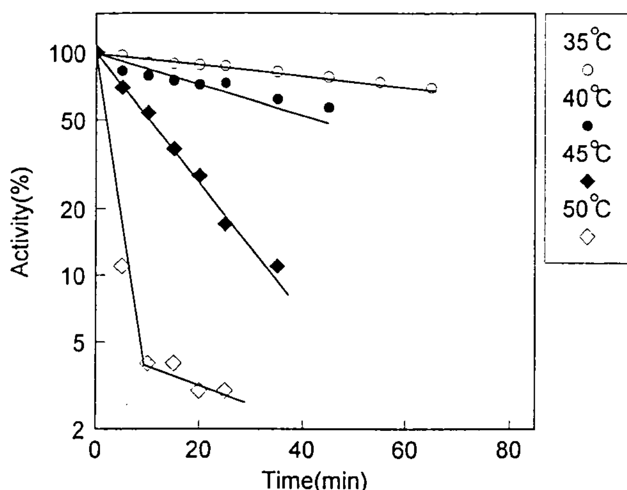


**Fig. 4.** Time dependent thermal inactivation of D-HIV dehydrogenase. The kinetic of time dependent thermal inactivation of D-HIV dehydrogenase was studied by incubation of D-HIV dehydrogenase at various temperatures and by the measurements of its residual activities.

the reaction components. For the investigation of thermal stability the mixtures were preincubated at several temperature for 10 min and the residual activities were measured. The enzyme is almost stable for 10 min at low temperature from 4℃ to 35℃. After incubation at 45℃ for 10 min the enzyme lost 50% of its activity and the activity was completely lost (95%) in 10 min at 50℃ as the result of increasing rate of thermal denaturation.

The kinetic of time dependent thermal inactivation of D-HIV dehydrogenase was studied based on these results (Fig. 4). D-HIV dehydrogenase was incubated at various temperatures and its residual activities were measured every 5 min. The half-times of inactivation ($t_{1/2}$) and the velocity constants of thermal inactivation were calculated from the initiation phase as shown in Table 3. The Arrhenius equation and the equation of Eyring were used for the calculation of activation energy, activation enthalpy and activation entropy of thermal inactivation of D-HIV dehydrogenase (Eyring, 1935).

● **Arrehenius equation:**

$$\log K = \log A - \frac{E_A}{2.303RT}$$

A : preexponential factor
$E_A$ : activation energy in $kJ \cdot mol^{-1}$
R : molar gas constant ($8.3144\ JK^{-1} \cdot mol^{-1}$)
T : temperature in K

● **The equation of Eyring:**

$$\log \frac{k}{T} = -\frac{\Delta H}{(19.147\ JK^{-1} \cdot mol^{-1})T} + \frac{\Delta S}{(19.147\ JK^{-1} \cdot mol^{-1})} + 10.319$$

k : velocity constant in $J \cdot mol^{-1}$
T : temperature in K
$\Delta H$ : activation enthalpy in $kJ \cdot mol^{-1}$
$\Delta S$ : activation entropy in $J \cdot mol^{-1} \cdot K^{-1}$

**Table 2.** Substrate spectra of D-HIV dehydrogenase for the reductive and oxidative reaction.

(a) Reductive reaction

| Substrate | $K_m$ (mM) | $V_{max}$ (mmol/min·mg) | $K_i$ (mM) |
|---|---|---|---|
| Pyruvate | – | – | – |
| 2-Ketobutyrate | – | – | – |
| 2-Ketovalerate | 4.2 | 2.314 | – |
| 2-Ketoisovalerate | 0.188 | 8.814 | – |
| 2-Ketocapronate | 4.4 | 0.926 | – |
| 2-Ketoisocapronate | 5 | 0.611 | – |
| 2-Keto-3-methyl-valerate | 0.4 | 1.851 | – |
| 2-Ketooctanate | – | – | – |
| NADPH | 0.333 | 9.740 | 0.4 |

(b) Oxidative reaction

| Substrate | $K_m$ (mM) | $V_{max}$ (mmol/min·mg) | $K_i$ (mM) |
|---|---|---|---|
| D-Hydroxybutyrate | – | – | – |
| D-Hydroxyisobutyrate | – | – | – |
| D-Hydroxyvalerate | – | – | – |
| D-Hydroxyisovalerate | 1.667 | 0.407 | – |
| D-Hydroxycapronate | – | – | – |
| D-Hydroxyisocapronate | – | – | – |
| D-Hydroxy-3-methyl-valerate | 6.700 | 0.648 | – |
| $NADP^+$ | 0.538 | 0.407 | 1.042 |

– : unmeasurable.

**Table 3.** The half-times ($t_{1/2}$) and the velocity constants of D-HIV dehydrogenase for the thermal inactivation

| T (℃) | $t_{1/2}$ (min) | k ($sec^{-1}$) |
|---|---|---|
| 35 | 87 | $1.33\times10^{-4}$ |
| 40 | 31.8 | $3.63\times10^{-4}$ |
| 45 | 6.7 | $1.72\times10^{-4}$ |
| 50 | 2.2 | $5.25\times10^{-4}$ |

From the Arrhenius diagram (Fig. 5, a) the activation energy of thermal inactivation was calculated as 209.2 kJ/mol. In the same way activation enthalpy and activation entropy could be determined by Eyring diagram as 206.4 kJ/mol and 0.35 kJ/mol·K, respectively (Fig. 5, b). The thermal inactivation of enzyme usually can be regarded as denaturation process of protein followed by considerable increase of entropy (Stern, 1949). the breaking of a few hydrogen bonds leads to the inactivation of enzymes. According to Stern, 21 kJ/mol of activation enthalpy is needed to break a hydrogen

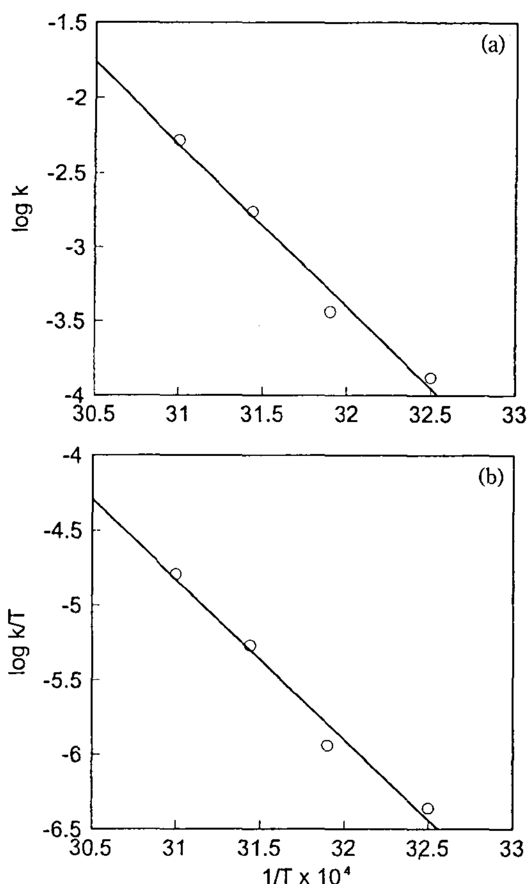


**Fig. 5.** Arrhenius and Eyring diagrams. (a) Arrhenius diagram (logk *vs.* 1/T), (b) Eyring diagram (log(k/T) *vs.* 1/T).

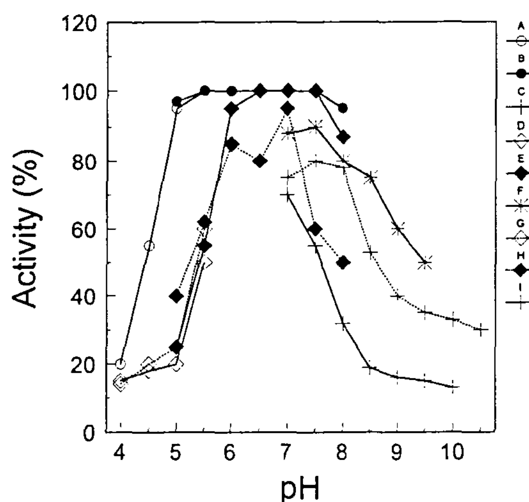


**Fig. 6.** Stability of D-HIV dehydrogenase in different buffer systems. D-HIV dehydrogenase stability during storage was studied for reductive reaction by using three buffer systems for 1 h at 25℃, 1 day at 4℃ and 3 months at −70℃. Aliquots of purified enzyme were stored in corresponding buffers. After incubation the preparations were transferred back into assay buffer and reactions were carried out immediately. A: 50 mM sodium acetate buffer (pH 4~5.3), 3 months at −80℃; B: 50 mM potassium phosphate buffer (pH 5~8), 3 months at −80℃; C: 50 mM Tris-HCl buffer (pH 7~11), 3 months at −80℃; D: 50 mM soium acetate buffer (pH 4~5.3), 1 h at 25℃; E: 50 mM potassium phosphate buffer (pH 5~8), 1 h at 25℃; F: 50 mM Tris-HCl buffer (pH 7~11), 1 h at 25℃; G: 50 mM sodium buffer (pH 4~5.3), 1 day at 4℃; H: 50 mM potassium phosphate buffer (pH 5~8), 1 day at 4℃; I: 50 mM Tris-HCl buffer (pH 7~11), 1 day at 4℃.

bond and is folowed by a gain of 50 J/mol·K of entropy by the breakage of bond. From this fact it can be supposed that 7 to 10 hydrogen bonds of D-HIV dehydrogenase were broken by thermal processes. In many cases the activation enthalpies and entropies of thermal inactivation are between 148 and 828 kJ/mol and between 0.1 and 2.3 kJ/mol·K (Stern, 1949). Activation enthalpy and activation entropy values of D-HIV dehydrogenase are within in this range and it means the thermal inactivation of D-HIV dehyrogenase was also due to the break of some hydrogen bonds in the enzyme structure.

## pH-stability of D-HIV dehydrogenase

The stability of D-HIV dehydrogenase during storage was studied for reductive reaction in three buffer systems under different pH conditions for 1 h at 25℃, 1 day at 4℃ and 3 months at −70℃. Aliquots of purified enzyme were stored in buffers at various pH. Fig. 6 demonstrates that D-HIV dehydrogenase activity was fairly stable between pH 6 and 8 in every case, and below 6 very unstable even though it was stored at −70℃. Above pH 8 it showed slightly different stability with temperature and incubation time. As can be seen in Fig. 6, potassium phosphate buffer was better than Tris-HCl buffer for storage of D-HIV dehydrogenase and optimal stability of the enzyme was observed in solution of pH values between 6 and 8. Routinely, small aliquots of the purified D-HIV dehydrogenase were stored at −70℃ in potassium phosphate buffer (pH 7.0) with 10% glycerol and thawed prior to use.

## Effect of various compounds on activity of the enzyme

The influence of reducing agents was investigated using N-ethylmaleimide, iodoacetamide and *p*-chloromecuribenzoate at concentrations up to 50 mM in the standard enzyme assay. These thiol blocking reagents were added to the enzyme solution and preincubated for 1 min at 35℃. The reaction was started by the addition of both substrates. Iodoacetamide, N-ethylmaleimide and *p*-chloromecuribenzoate inhibited the enzyme activity about 80% at the concentrations of 0.02 mM, 50 mM and 50 mM, respectively. These results mean that the thiol group in the active center of the enzyme might play an important role in catalysis. The

effects might also explain the fact that dithioerythritol had to be added to the all steps during the purification (Lee *et al.*, 1992).

The effects of salt ions on the activity of the enzyme were tested in the standard assay system using KCl, NaCl, and $(NH_4)_2SO_4$ in concentration up to 100 mM. The activity of D-HIV dehydrogenase was strongly inhibited by ionic strength over the concentration of 30 mM compared to the effect of potassium phosphate buffer in which that of enzyme was inhibited by over 300 mM of buffer itself (data not shown). The enzyme activity was reduced by various metal ions at 0.1 mM, such as $Fe^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ to 33%, 0%, 12%, 24%, and 91%, respectively. Therefore during all purification steps EDTA must be added to avoid the loss of enzyme activity.

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