The differential scanning calorimetry analysis of the murine major histocompatibility complex class II molecule, I-E\(^k\), in complex with an antigenic peptide derived from mouse hemoglobin, showed that the thermal stability at the mildly acidic pH is higher than that at the neutral pH. Although the thermal unfolding of I-E\(^k\)-hemoglobin was irreversible, we extracted the equilibrium thermodynamic parameters from the kinetically controlled heat capacity curves. Both the denaturation temperatures and the enthalpy changes were almost independent of the heating rate over 1 °C per min. The linear relation between the denaturation temperature and the calorimetric enthalpy change provided the heat capacity changes, which are classified into one for the mildly acidic pH region and another for the neutral pH region. The equilibrium thermodynamic parameters showed that the increased stability at the mildly acidic pH is because of the entropic effect. These thermodynamic data provided new insight into the current structural features.

Thus, I-E\(^k\) may be a good target for further thermodynamic characterizations and for studying their relation to the structural features.

DSC analyses can provide accurate thermodynamic parameters, such as the calorimetric enthalpy change (\(\Delta H_{\text{cal}}\)) and the entropy change (\(\Delta S\)), in addition to the denaturation temperature (\(T_d\)) and the van’t Hoff enthalpy change (\(\Delta H_{\text{vH}}\)), which are also obtained in circular dichroism (CD) measurements. It is interesting to determine the energetic contribution to the increased stability of MHC class II molecules at low pH relative to that at neutral pH (10), which can explain the structural and functional differences. Additionally, to analyze the irreversible thermal denaturation of I-E\(^k\)-Hb, the kinetically controlled heat capacity functions were used for the extraction of equilibrium parameters (17). Because reversible denaturation is limited to small, compact proteins, the evaluation of irreversible transitions will be more important to analyze structure-func-

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§ The abbreviations used are: MHC, major histocompatibility complex; CLIP, class II associated invariant chain-derived peptide; Hb, hemoglobin; DSC, differential scanning calorimetry; \(\Delta H_{\text{cal}}\), calorimetric enthalpy change; \(\Delta S\), entropy change; \(T_d\), denaturation temperature; MOPS, 3-(N-morpholino)propanesulfonic acid; \(\Delta H_{\text{vH}}\), van’t Hoff enthalpy change; \(C_e\), molar excess heat capacity; \(\Delta C_p\), molar excess heat capacity change; \(E_a\), activation energy of irreversible unfolding; \(T^*\), temperature at which the rate constant from denatured to irreversibly arrived state is unity; \(\Delta G(T)\), Gibbs free energy of unfolding as a function of temperature.

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* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to
tion relationships in the present post-genome era. Under the same conditions used for DSC measurements, with little interference from the irreversible process, the thermodynamic parameters can be quantitatively determined and compared as a function of pH.

EXPERIMENTAL PROCEDURES

Expression and Purification of MHC Class II—Baculovirus-infected insect cells, Sf9, were used to produce soluble I-Eκ molecules with the Hb peptide, 64–76, linked to the N terminus of the β subunit via flexible linker, as described previously (18, 19). The I-Eκ-Hb molecule was secreted into the medium and was purified by immunoaffinity column chromatography with 14-4-4S, a monoclonal anti-MHC class II antibody, followed by gel filtration chromatography using a Superdex 200 column (16 mm × 80 cm, Amersham Biosciences). The purified fractions were pooled, and the buffer was exchanged to an appropriate buffer.

The protein concentrations were determined from UV absorption at 280 nm and were calculated by using an absorption coefficient of 1.23 cm⁻² mg⁻¹, which was estimated from the amino acid composition of I-Eκ-Hb.

CD Measurements—Far-UV CD spectra of I-Eκ-Hb were measured on a Jasco J-600 spectropolarimeter. The protein concentration was 0.36 mg/ml, and the optical path length was 0.1 cm. Spectra for CD between 200 and 250 nm were obtained in 10 mM phosphate buffer containing 150 mM NaCl at 20°C using a scanning speed of 10 nm min⁻¹, a time response of 2 s, a band width of 1 nm, and an average over 5 scans.

DSC Measurements—DSC experiments were carried out on a Microcal MCS DSC calorimeter. All solutions (10 mM phosphate buffer containing 150 mM NaCl or 10 mM MOPS buffer containing 150 mM NaCl) were carefully degassed before the measurements. Data were collected in the temperature range between 20 and 90°C at various heating rates, 0.2, 1.0, 1.5, and 2.0 °C min⁻¹. The protein concentration was in the range from 0.08 to 0.43 mg ml⁻¹, a time response of 2 s, a band width of 1 nm, and an average over 5 scans.

The temperature dependence of the first-order rate constant, k₂, is given by the Arrhenius equation, the Hb peptide was attached by a flexible linker to the A rate-limiting irreversible

\[ k_2 = A \exp\left(-\frac{E_a}{RT}\right) \]  

(Eq. 6)

Method B—The variation of T_d with r is given by the following equation.

\[ \frac{r}{(T_d)^{1/2}} = \frac{AR}{E_a} \exp\left(-\frac{E_a}{RT}\right) \]  

(Eq. 7)

Method C—The dependence of the enthalpy evolved with temperature is expressed by the following equation.

\[ \ln[\frac{E_a}{(\Delta H_{1002})}] = \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_d} \right) \]  

(Eq. 8)

Method D—The activation energy can be calculated from the heat capacity at T_d, C_p(T_d), by the following equation.

\[ E_a = \frac{c_p(T_d)}{\Delta H_{1002}} \]  

(Eq. 9)

RESULTS

CD Analysis of I-Eκ-Hb as a Function of pH—To eliminate the binding of endogenous peptides to I-Eκ during its expression, the Hb peptide was attached by a flexible linker to the N terminus of the I-Eκ β subunit, and the soluble molecule, I-Eκ-Hb, was purified as a single peak on gel filtration high performance liquid chromatography analysis, corresponding to the αβ heterodimer (Fig. 1A). The SDS-PAGE analysis also revealed the purity of the I-Eκ-Hb molecule to be over 95%. The far-UV CD spectra of I-Eκ-Hb were similar to those of I-Eκ, reported previously (12), and showed that the secondary structure was not grossly altered as a function of pH (Fig. 1B).

Thermal Denaturation Analysis of I-Eκ-Hb as a Function of pH—For DSC measurements at mildly acidic and neutral pH

Increased Stability of MHC Class II at Acidic pH

**TABLE I**

Thermodynamic parameters for denaturation of I-E\(^2\)-Hb as a function of pH

| pH | Concentration | \(T_d\) | \(\Delta H_{\text{cal}}\) | \(\Delta H_{\text{th}}\) | \(\Delta H_{\text{cal}}/\Delta H_{\text{th}}\) |
|----|---------------|--------|-----------------|-----------------|-----------------|
| 5.0 | 0.34          | 67.2   | 651             | 565             | 1.2             |
| 5.5 | 0.33          | 70.6   | 682             | 652             | 1.2             |
| 6.0 | 0.32          | 73.6   | 702             | 665             | 1.1             |
| 6.5 | 0.33          | 74.9   | 718             | 665             | 1.1             |
| 7.0 | 0.33          | 72.8   | 713             | 652             | 1.2             |
| 7.4 | 0.33          | 67.2   | 744             | 600             | 1.2             |
| 8.0 | 0.33          | 62.7   | 651             | 565             | 1.2             |

The heating rate was 1.0 °C per min.

**TABLE II**

Thermodynamic parameters for denaturation of I-E\(^2\)-Hb in MOPS buffer

| pH | Concentration | \(T_d\) | \(\Delta H_{\text{cal}}\) |
|----|---------------|--------|-----------------|
| at 25 °C | at \(T_d\) | mg ml\(^{-1}\) | °C |
| 6.5 | 5.5 | 0.33 | 72.6 |
| 7.0 | 6.0 | 0.33 | 73.6 |
| 7.4 | 6.5 | 0.33 | 70.6 |
| 7.7 | 7.0 | 0.50 | 68.5 |

The heating rate was 1.0 °C per min.

**TABLE III**

Thermodynamic parameters for denaturation of I-E\(^2\)-Hb at pH 7.4 as a function of protein concentration

| Concentration | \(T_d\) | \(\Delta H_{\text{cal}}\) | \(\Delta H_{\text{th}}\) | \(\Delta H_{\text{cal}}/\Delta H_{\text{th}}\) |
|---------------|--------|-----------------|-----------------|-----------------|
| 0.08 mg ml\(^{-1}\) | 66.9 | 744 | 608 | 1.2 |
| 0.18 | 65.9 | 756 | 593 | 1.3 |
| 0.33 mg ml\(^{-1}\) | 67.2 | 744 | 600 | 1.2 |

* Data were taken from Table I.

values, the I-E\(^2\)-Hb molecule was first dissolved in phosphate buffer, because of its lower enthalpy change for the deprotonation and its lower temperature dependence (21). Fig. 2 shows the excess heat capacity curves as a function of pH, at a heating rate of 1 °C per min. All curves were irreversible as shown by the lack of reproduced excess heat capacity in the second scanning, as described below. The small transition ranges of about 10 °C indicated the high cooperativity of this transition. Assuming the two-state transition, the thermodynamic parameters for denaturation of I-E\(^2\)-Hb were determined, and are summarized in Table I. It should be noted that the stability at the mildly acidic pH is higher than that at the neutral pH. The correlation between \(T_d\) and \(\Delta H_{\text{cal}}\) could be classified into two groups, one for the mildly acidic pH and the other for the neutral pH (Fig. 3). The heat capacity change (\(\Delta C_p\)), determined from this correlation, is 11.1 kJ mol\(^{-1}\) K\(^{-1}\) for the mildly acidic pH and that of the neutral pH is 15.9 kJ mol\(^{-1}\) K\(^{-1}\).

To evaluate the protonation effects on the buffer and the protein (22, 23), the thermodynamic stability of I-E\(^2\)-Hb, in a buffer with a large heat of ionization, MOPS, was also analyzed using DSC. Because the pH of MOPS buffer is largely dependent on the temperature (21), the pH at the \(T_d\) of the buffer used in this study was determined and applied to compare the thermodynamics in the phosphate buffer described above. Table II summarizes the thermodynamic parameters obtained in MOPS buffer. The thermal stability at an acidic pH is higher than that at a neutral pH, which is similar to the stability in the phosphate buffer. The \(\Delta H_{\text{cal}}\) values in MOPS buffer are relatively smaller than those in phosphate buffer. This difference should be because of the protonation effects on the buffer and the protein.
heating rates, 0.2, 1.0, 1.5, and 2.0 °C per min, at pH 5.5 and 7.4, to analyze the kinetically controlled denaturation of I-Ek-Hb (Fig. 5). The thermodynamic parameters obtained from each transition curve are summarized in Table IV. Whereas the $T_d$ value at the rate of 0.2 °C per min was significantly lower than the others, those at the rate over 1 °C per min were similar at both pH values, indicating that the transition temperatures at high heating rates reach their maximum values with little effect from the irreversibility. All of the $\Delta H_{cal}$ values obtained with the various heating rates were similar, within experimental error.

**Activation Parameters of Irreversible Denaturation of I-Ek-Hb**—Sanchez-Ruiz et al. (17) proposed four methods to evaluate the activation energy from the excess heat capacity curves, using the scan rate dependence of irreversible denaturation. The graphical presentations of the evaluations according to methods A, B, and C, described under “Experimental Procedures,” are shown in Fig. 6, and the analyzed activation parameters, including method D, are summarized in Table V. Although the activation energies derived from the various methods differed, the average value was larger than the previously reported values for the denaturation of other proteins.

### Table IV

| pH  | Heating rate (°C min$^{-1}$) | $T_d$ (°C) | $\Delta H_{cal}$ (kJ mol$^{-1}$) | $\Delta H_{th}$ (kJ mol$^{-1}$) | $\Delta H_{cal}/\Delta H_{th}$ |
|-----|----------------------------|-----------|---------------------------------|-------------------------------|-------------------------------|
| 5.5 | 0.2                        | 71.6      | 766                             | 647                           | 1.2                           |
|     | 1.0                        | 75.4      | 723                             | 702                           | 1.0                           |
|     | 1.5                        | 76.9      | 656                             | 727                           | 0.9                           |
|     | 2.0                        | 77.8      | 618                             | 725                           | 0.9                           |
| 7.4 | 0.2                        | 60.4      | 650                             | 571                           | 1.1                           |
|     | 1.0                        | 67.2      | 744                             | 600                           | 1.2                           |
|     | 1.5                        | 67.4      | 705                             | 579                           | 1.2                           |
|     | 2.0                        | 66.2      | 775                             | 556                           | 1.4                           |

**Fig. 4.** A series of repeated heating and cooling steps of I-Ek-Hb at pH 5.5 (A) and 7.4 (B). A, the scans were stopped at 65 (1), 70 (2), 73 (3), 75 (4), and 80 °C (5). Curve 6 is the second scan after the full scan, and curve 7 is the full scan, taken from Fig. 2. B, the scans were stopped at 50 (1), 55 (2), 60 (3), 63 (4), 65 (5), 70 (6), and 80 °C (7). Curve 8 is the second scan after the full scan, and curve 9 is the full scan, taken from Fig. 1. The heating rate was 1.0 °C per min.

**Fig. 5.** Variation with heating rate of I-Ek-Hb unfolding at pH 5.5 (A) and 7.4 (B). The heating rate is indicated at each transition curve. The protein concentration for the respective measurements was 0.4 mg/ml.
which are in the range between 280 and 360 kJ mol$^{-1}$ (24). The large activation energy suggests that most of the native structure of I-E$^b$-Hb should be denatured before the irreversible transition occurs, and the thermodynamic parameters for the denaturation of I-E$^b$-Hb could be analyzed as an equilibrium quantity, as described above. Similar to the activation energy, the T*$*$ values were almost independent of the scan rate, and were higher than the T$_d$ values, except for those from the scan rate of 2 °C per min (Tables IV and V). Together with the results of the scan rate experiments, this result supports the validity of the approximation to determine the equilibrium thermodynamics from DSC experiments with the scan rate of 1 °C per min.

**DISCUSSION**

Irreversible thermal denaturation is observed in many proteins, including MHC molecules, which makes it difficult to analyze the precise thermodynamics. In the present study, we found the interesting phenomenon of MHC stability as a function of pH, although the thermal denaturation process was irreversible. The thermodynamic origin of the increased stability at the mildly acidic pH could be because of the dynamic
properties of MHC molecules, which are important for the function of MHC class II molecules. To determine the thermodynamics and to evaluate their validity, the effects of the irreversibility should first be analyzed under various conditions. Because the method to extract thermodynamic equilibrium parameters from kinetically controlled heat capacity curves has been applied successfully to several systems (17, 24, 25), we used this method in the present study. The \( T_d \) and \( \Delta H \) values were almost independent at a protein concentration of around 0.3 mg ml\(^{-1}\) and a heating rate over of 1 °C per min (Tables III and IV). Additionally, the large activation energy and the high \( T^* \) value indicated that the thermal denaturation process under these conditions could be analyzed as the equilibrium thermodynamics, with little interference from the irreversible process (Table V). Therefore, the thermodynamic parameters as a function of pH (Table I), obtained with a heating rate of 1 °C per min and a protein concentration of about 0.3 mg ml\(^{-1}\) are meaningful.

In the previous CD analyses of the thermal denaturation of MHC class II molecules, the temperature was increased in a stepwise mode (10, 11). Because it takes a few minutes at each temperature for equilibrium attainment and recording, the \( T_d \) values should be lower than those obtained with the method of continuous heating used in this study. This is supported by the fact that the stability of I-E\(^k\)-Hb at pH 7.4 in the previous CD measurements was similar to that at the heating rate of 0.2 °C per min in this study (Table IV) (11). Under the conditions of a stepwise mode or a low heating rate for the analysis of irreversible denaturation, the \( T_d \) value is largely dependent on the heating rate.

In the thermal denaturation process of I-E\(^k\)-Hb, three transitions should be involved: 1) peptide dissociation from the MHC class II molecule, 2) dissociation of the \( \alpha \beta \) heterodimer to each subunit, and 3) denaturation of each subunit. The excess heat capacity curve of I-E\(^k\)-Hb should be the sum of these transitions. Although the ratio \( \Delta H_{\text{obs}}/\Delta H_{\text{olv}} \) under the various conditions of the DSC measurements is around 0.9 to 1.4 (Tables I, III, and IV), we cannot exclude the possibility of the existence of intermediate states and/or the coupling of respective transitions. Because the bound peptide contributes to the stability of MHC molecules (26), the peptide dissociation should occur first, to cause the subsequent denaturation. This is also supported by the results that the thermal denaturation profiles of I-E\(^k\) in complex with mutant peptides of Hb seemed to be cooperative, similar to that of I-E\(^k\)-Hb, although their \( T_d \) values differed from each other.\(^2\) These results indicate that the apparent stability of the MHC-peptide complex is largely dependent on the binding kinetics and/or the affinity of the bound peptide. The empty MHC class II molecule, I-A\(^k\), in complex with CLIP and the antigenic peptide derived from the \( \alpha \) subunit of the I-E molecule, E\(_\alpha\) (28). In contrast, the thermal stabilities of I-A\(^k\)-CLIP and I-E\(^k\) complexed with the moth cytochrome c peptide at a mildly acidic pH are similar to those at neutral pH (10). Despite the stability difference at both pH regions, all of the MHC class II molecules are resistant to a lower pH, that is in contrast to the stability of the MHC class I molecule (10). The increased stability at a mildly acidic pH of the other MHC class II molecules may also be because of entropic effects, similar to I-E\(^k\)-Hb as described below, which generally correlates with their functions in an acidic compartment.

In addition to the difference in the \( T_d \) values, other thermodynamic parameters for denaturation at the mildly acidic pH differed from those at the neutral pH. The correlation of \( T_d \) with \( \Delta H_{\text{cal}} \) at various pH values indicated that the folding of I-E\(^k\)-Hb could be classified into two groups (Fig. 3), which is consistent with the difference observed in the structural analyses, as described below. The \( \Delta C_p \) values obtained from the slope of \( T_d \) versus \( \Delta H_{\text{cal}} \) in phosphate buffer were 11.1 kJ mol\(^{-1}\) K\(^{-1}\) for the mildly acidic pH and 15.9 kJ mol\(^{-1}\) K\(^{-1}\) for the neutral pH. To compare the obtained values with those of other proteins with a similar size, the empirical method proposed by Oobatake and Ooi (29) was applied and the \( \Delta C_p \) value of a 420-residue (NR) protein was calculated to be 30.2 kJ mol\(^{-1}\) K\(^{-1}\) from the following equation.

\[
\Delta C_p (\text{cal mol}^{-1} \text{ K}^{-1}) = -512 + 18.39 \text{NR} \quad \text{(Eq. 10)}
\]

This value, 30.2 kJ mol\(^{-1}\) K\(^{-1}\), is larger than the experimentally determined values of I-E\(^k\)-Hb at either pH region, indicating that the surface of non-polar residues of I-E\(^k\)-Hb is more accessible to the solvent than that of other proteins. Furthermore, it should be noted that the \( \Delta C_p \) value at the mildly acidic pH is lower than that at the neutral pH. This is consistent with the previous results, in which the acidification changed I-E\(^k\) into a more fluctuating state with an increase in the exposed hydrophobicity, like a molten globule state (12, 13).

The analyses of the protonation effects showed that the \( \Delta H_{\text{cal}} \) values in the buffer with a larger heat of ionization, MOPS, are smaller than those in the buffer with a lower heat of ionization, phosphate, whereas the thermal stability at an acidic pH is higher than that at a neutral pH. The difference of the \( \Delta H_{\text{cal}} \) values should be because of the enthalpy of buffer ionization and the linked protonation effects (21, 22). Petrosian and Makhatadze (23) successfully evaluated the contribution of linked protonation effects on the stability of a 69-amino acid residue protein, with consideration of the isoelectric point (pI). In the case of a 420-residue protein, I-E\(^k\)-Hb, the pI value in the denatured state is estimated to be 4.8 from the amino acid composition, and that in the native state is estimated to be 5.0 by the isoelectric focusing experiments (data not shown). Amino acid residues such as Asp and His, with charges that are affected at the pH region analyzed in this study, are thought to be involved in the structural and functional characters of MHC class II molecules (16, 30, 31).

Therefore, the present thermodynamic results obtained in phosphate buffer, which has lower enthalpy change for the deprotonation, could be the comparable data to analyze the effects of pH on the stability difference of I-E\(^k\)-Hb. Assuming the enthalpy derived from linked protonation effects is compensated by the large ionization enthalpy of MOPS buffer, as observed in the CspA unfolding (23), the conformational en-

\(^2\) K. Saito, A. Sarai, M. Oda, T. Azuma, and H. Kozono, unpublished results.

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### Table VI

| pH | \( T_d \) \( ^\circ \text{C} \) | \( \Delta T_d \) | \( \Delta H \) | \( T \Delta S^\text{obs} \) | \( \Delta G \) |
|----|----------------|-------------|----------|----------------|-----|
| 5.5 | 75.4 | 723 | 723 | 0 |
| 7.4 | 67.2 | -8.2 | 874 | 885 | -11 |

\( T_d \) values were taken from Table I. \( T_d \) values were calculated from the equation, \( \Delta G(T) = \Delta H(T) - T \Delta S(T) \).
enthalpy change can be estimated to be about 700 kJ/mol. Still on this assumption, the increased stability of I-Ek-Hb at acidic pH is because of a favorable $\Delta S$, as described below.

To analyze the thermodynamic origin of the stability difference, the thermodynamic parameters at 75.4 °C, the denaturation temperature at pH 5.5, were calculated using the correlation of $\Delta G$ and $\Delta H$ with temperature (Table VI). Within the narrow range of temperature around the $T_d$ values, the errors of the calculated $\Delta G$ and $\Delta H$ values at the reference temperature should be small, even if the $\Delta C_p$ values used contain some errors, because of the temperature dependence and the linked protonation effects. This result clearly indicates that the higher stability at the mildly acidic pH than that at the neutral pH is because of the difference in the entropic contributions. One possible explanation for this entropic difference as a function of pH is that the native structure of I-Ek-Hb at the mildly acidic pH is more flexible, which can facilitate the peptide exchange. This is consistent with the previous SDS-PAGE and structural analyses, in which I-Ek gained flexibility at low pH (11, 14).

The smaller $\Delta C_p$ value of I-Ek-Hb at low pH, as described above, should also be because of this increased flexibility. Although the difference in the secondary and tertiary structures between the mildly acidic and neutral pH values detected in the CD measurements is subtle (Fig. 1B), similar to the previous reports (12, 32), the molecule will be more dynamically fluctuating and the surface of non-polar residues will be more accessible to solvent at a mildly acidic pH.

The kinetic and structural analyses of I-Ek-Hb have shown that the hydrogen bonding network, formed by the cluster of carboxylate groups around the P6 pocket, Asp$^{66}$ and Glu$^{11}$ of the $\alpha$ subunit, Asp$^{73}$ of the Hb peptide, and water molecules, changes as a function of pH, which can regulate the conformation of I-Ek and the peptide exchange rate (11). The protonation of these carboxylate groups at low pH, together with other residues such as His$^{33}$ of the $\beta$ subunit, facilitates the flexibility and the conformational change of the MHC class II molecule from the closed to the open form (30). In addition, the recent mutational analyses of HLA-DR, the human homologue of I-Ek, have shown that His$^{33}$ of the $\alpha$ subunit has the role of a pH-sensitive switch at low pH, which can regulate the conformational transition and the peptide exchange (31). The decreased $\Delta H$, $T\Delta S$, and $\Delta C_p$ values for the thermal denaturation of I-Ek-Hb at the mildly acidic pH relative to those at the neutral pH should be because of the differences in the dynamic properties, which are closely related with the function of MHC class II molecules. The present DSC analyses provide thermodynamic insight into the increased stability at the acidic pH.

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Thermodynamic Analysis of the Increased Stability of Major Histocompatibility Complex Class II Molecule I-E\(^k\) Complexed with an Antigenic Peptide at an Acidic pH

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