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Chemical Enthalpy–Entropy Compensation Effect in the Hydrolysis of *p*-Nitrophenyl Carboxylates Catalyzed by Alkaline Mesentericopeptidase

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The temperature dependence of the hydrolysis of *p*-nitrophenyl carboxylates with general formula $H(CH_2)_nCOOC_6H_4NO_2$ catalyzed by alkaline mesentericopeptidase has been studied (*n* varying from 1 to 7, temperature range 2–30°C, pH 8.80, 5 vol% dimethylsulfoxide). The activation parameters of the deacylation step depend on the length of the hydrophobic side chain of the substrate molecule ($\Delta G_3^{\neq}$, $\Delta H_3^{\neq}$, and $\Delta S_3^{\neq}$ decrease by 2.0 kcal/mol, 4.9 kcal/mol, and 10 eu, respectively, as the length of the acyl carbon chain increases from $n = 1$ to $n = 4$). The following criteria were applied to establish a chemical enthalpy–entropy compensation effect: (a) Exner's plot of $\log k_3^{T_1}$ vs $\log k_3^{T_2}$: (b) Petersen's plot of $\log k/T$ vs $1/T$; (c) Exner's statistical treatment in coordinates $\log k$ vs $1/T$; (d) according to Krug *et al.* ($\Delta H^{\neq}$ vs $\Delta G^{\neq}_{T_{hm}}$). By use of all the above-mentioned criteria the existence of a chemical enthalpy–entropy compensation effect was proved with an isokinetic temperature β of about 470°K, which is significantly higher than the average experimental temperature.

INTRODUCTION

The enthalpy–entropy compensation in enzyme reactions has been discussed for many years (*1*, *2*). Two general problems arise upon studying this phenomenon: first, does the ΔH vs ΔS linear relationship have a chemical origin or is it an artifact; and second, what is the source of the compensation effect? From the experimental data and theoretical corollaries accumulated so far, no satisfactory explanation has been offered. As Exner (*3*) and Krug *et al.* (*4*) have pointed out, many of the reports dealing with this effect and especially with the values of the "isokinetic" or the "compensation" temperature are of no great significance due to improper statistical treatment of the experimental data.

In the present paper the effect of temperature on the deacylation step of the alkaline mesentericopeptidase-catalyzed hydrolysis of *p*-nitrophenyl carboxylates is reported in order to evaluate the dependence of the activation parameters on the structure of the substrates. An attempt to test the chemical compensation effect is made using all criteria available.

The specificity of alkaline mesentericopeptidase in terms of activation free energy toward *p*-nitrophenyl carboxylates has already been reported (*5*). For further elucidation of the kinetic specificity of this enzyme as well as that of other

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enzymes, a study of the dependence of enthalpy and entropy of activation on the substrate structure appeared desirable. In several cases it has been found that these parameters depend on the size of the hydrophobic side chain (*6, 7*). But in none of these studies was a correct statistical treatment of the experimental data made.

EXPERIMENTAL

Materials. Alkaline mesentericopeptidase (EC 3.4.21.-) was isolated in a crystal state by the method described previously (*8*). Enzyme stock solutions were made up in 0.7 *M* $CaCl_2$ (*9*). The enzyme preparation was 80% pure with respect to active enzyme as determined by spectrophotometric titration of the active sites by *N-trans*-cinnamoylimidazole (*10*). *p*-Nitrophenyl esters of aliphatic carbon acids were synthesized from the corresponding chlorides (*11, 12*). Substrate solutions were made up in freshly distilled dimethylsulfoxide.

Kinetic measurements. The reaction rate was measured by following the appearance of *p*-nitrophenol. Under experimental conditions (0.02 *M* borate buffer, pH 8.80, 0.1 *M* KCl, 5 vol% dimethylsulfoxide) the extinction coefficient at 400 nm was $\epsilon = 1.75 \times 10^4 M^{-1}\,cm^{-1}$ and is temperature and pH independent. As it was found in the present work and in (*13*), the pH dependence of the hydrolysis of the reaction series catalyzed by alkaline mesentericopeptidase has a sigmoidal form with a pK near 7. As the investigations were carried out in the pH-independent region of the pH profile, the negligibly small variations of pH caused by the temperature changes did not affect the rate constants. Kinetic data were obtained in a Specord UV VIS spectrophotometer within the temperature interval 2–30°C. The temperature was maintained constant (±0.05°C) during the kinetic runs by means of a Ultracriostat – MK 20 (GDR).

The concentration of enzyme in the reaction mixture varied from $4.4 \times 10^{-7} M$ to $6.7 \times 10^{-6} M$. The concentration of *p*-nitrophenyl esters varied in the following ranges: acetate, 5.7×10^{-4}–$1.4 \times 10^{-3} M$; propionate, 9.5×10^{-5}–$3.9 \times 10^{-4} M$; butyrate, 1.0×10^{-4}–$2.5 \times 10^{-4} M$; valerate, 7.6×10^{-5}–$1.9 \times 10^{-4} M$; hexanoate, 3.7×10^{-5}–$9.5 \times 10^{-5} M$; heptanoate, 1.8×10^{-5}–$4.5 \times 10^{-5} M$; octanoate, 9.0×10^{-6}–$2.3 \times 10^{-5} M$. The ratio $[S_0]/K_m$ varied from 0.1 to 1.5 for the different substrates and different temperatures.

In the reaction studied the hydrolysis of the acyl enzyme is rate limiting ($k_2 \gg k_3$). The reaction rate measured is described by the equation:

$$v = \frac{k_3[E_0][S_0]}{K_m + [S_0]} .$$

The condition $k_2 \gg k_3$ follows from the study of biphasic *p*-nitrophenol liberation. The "burst" of *p*-nitrophenol obtained by extrapolation to zero time is equal to the "burst" of *N*-transcinnamoylimidazole titration of enzyme. It does not depend on the nature and the concentration of the substrate ($[S_0] \gg [E_0]$) and is proportional to the enzyme concentration only.

A least-squares treatment of Lineweaver–Burk plots was applied to evaluate

the deacylation rate constants k_3. The activation parameters were calculated by the same procedure using coordinates log k_3 vs $1/T$.

RESULTS AND DISCUSSION

The activation free energy for the alkaline hydrolysis of ethyl (*14*) and *p*-nitrophenyl (*15*) carboxylates depends slightly on the length of the substrate side chain. The enthalpy of activation for the same reaction of ethyl carboxylates is practically constant, both in aqueous ethanol (*14*) and in aqueous acetone (*16, 17*). Unlike nonenzymatic reactions the hydrolysis of acyl enzymes is strongly dependent even on small changes in the substrate structure. The values of activation parameters for the deacylation step are presented on Table 1. The dependence of $\Delta G_3^{\neq}$, $\Delta H_3^{\neq}$, and $T\Delta S_3^{\neq}$ on the length of the substrate side chain is shown in Fig. 1. It can be seen that the enthalpy and entropy of activation change with the substrate structure in the same manner as the activation free energy, an extremum at $n = 4$ being observed. A similar dependence of the effectivness of the catalysis on the hydrophobicity of the side chain in the substrate molecule has been reported for α-chymotrypsin (*6*), elastase (*7*), trypsin (*18*), etc. Probably alkaline mesentericopeptidase, as with α-chymotrypsin and elastase, possesses in the binding site a hydrophobic cavity with small dimensions.

Criteria for Testing the Chemical Compensation Effect

The considerable enthalpy change supports the idea of a flexible active site. The lower sensitivity of $\Delta G_3^{\neq}$ in comparison with $\Delta H_3^{\neq}$ to the substrate structure implies the existence of an enthalpy–entropy compensation effect.

In the early papers a simple linear correlation between ΔH and ΔS was

TABLE 1

ACTIVATION PARAMETERS FOR THE DEACYLATION STEP IN THE ALKALINE MESENTERICOPEPTIDASE-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL CARBOXYLATES, $H(CH_2)_nCOOC_6H_4NO_2$[a]

n	$k_3 \times 10^2$ (sec^{-1})[b]	$\Delta G_3^{\neq}$ (kcal/mol)[b]	$\Delta H_3^{\neq}$ (kcal/mol)	$-\Delta S_3^{\neq}$ (eu)
1	6.5 ± 0.4[c]	18.94 ± 0.04	16.5 ± 0.9	8.0 ± 2.9
2	15.0 ± 0.6	18.46 ± 0.02	15.2 ± 0.5	11.1 ± 1.5
3	59.7 ± 2.4	17.66 ± 0.02	12.6 ± 0.3	17.1 ± 0.9
4	206 ± 4	16.96 ± 0.02	11.6 ± 0.4	18.1 ± 1.2
5	30.8 ± 0.6	18.02 ± 0.02	14.1 ± 0.8	13.8 ± 2.8
6	6.4 ± 0.2	18.98 ± 0.02	13.4 ± 0.6	18.8 ± 2.1
7	3.8 ± 0.2	19.29 ± 0.03	17.8 ± 0.3	5.0 ± 0.9

[a] pH 8.80, 0.1 *M* KCl, 5 vol% dimethylsulfoxide, temperature range 2–30°C.
[b] At 25°C.
[c] The stated uncertainties are standard deviation of the mean.

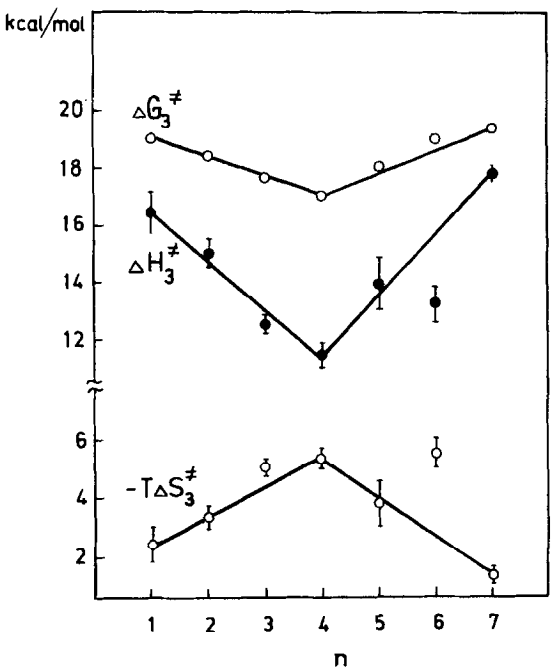

FIG. 1. Dependence of the activation parameters for the deacylation step in the alkaline mesenterico-peptidase-catalyzed hydrolysis of *p*-nitrophenyl carboxylates, $H(CH_2)_nCOOC_6H_4NO_2$, upon the length of the hydrophobic side chain. Conditions: pH 8.80, 0.02 *M* borate buffer, 0.1 *M* KCl, 5 vol% of dimethylsulfoxide.

considered as a proof of an isokinetic relationship. Because of the mutual dependence of these quantities obtained from a single equation and the narrow temperature interval used in studies of enzyme reactions, the ΔH vs ΔS plots are misleading, since in most cases they reveal a statistical rather than a chemical compensation effect (*4*).

According to Exner (*19*), a criterion for the existence of a compensation effect is the linear relationship of kinetic constants at two temperatures:

$$\log k_3^{T_1} = b \log k_3^{T_2} + \text{const.}$$

The slope b is related to the isokinetic temperature β by the equation:

$$\beta = T_1 T_2 \frac{b - 1}{bT_1 - T_2} \qquad (T_1 > T_2).$$

Since the values of $\log k_3^{T_1}$ and $\log k_3^{T_2}$ are determined by independent experiments, this treatment is statistically correct. The dependence of $\log k_3^{298°K}$ vs $\log k_3^{279°K}$ is shown in Fig. 2. This dependence is linear with a correlation coefficient $r = 0.998$ and $b = 0.852 \pm 0.056$ ($P = 95\%$). The good correlation implies the existence of an isokinetic relationship with an isokinetic temperature of $490 \pm 147°K$.

Because of a narrow temperature interval, usually the slope b is near unity and a

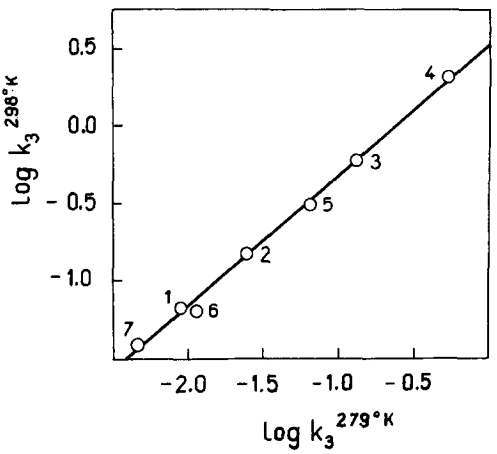

FIG. 2. Plot of log k_3 at two temperatures for the hydrolysis of *p*-nitrophenyl carboxylates catalyzed by alkaline mesentericopeptidase. (The indication of the points corresponds to *n*.)

large error can arise in determination of β. In this case a strict statistical treatment is necessary in order to estimate the difference between b and T_1/T_2 (*20*). The inequality $\beta > T_2$ is valid when $T_1/T_2 - b$ exceeds at least two to three times the standard deviation s of b. In our case $T_1/T_2 - b = 3.5s$.

Another criterion to test the compensation effect according to Petersen (*21*) is the existence of a common point of intersection of lines, corresponding to each member of the reaction series obtained in coordinates log k/T vs $1/T$. The abscissa of the intersection point corresponds to the isokinetic temperature. In our case an isokinetic temperature of about 500°K was found. It must be pointed out that, although this method is unobjectionable from a formal point of view, its graphical representation is inexact, because in most cases the isokinetic temperature is a product of extrapolation far from the experimental realizable temperatures.

More recently Exner (*3*) suggested a proper statistical treatment to test the compensation effect and to obtain the isokinetic temperature in Arrhenius coordinates. The statistical problem is to estimate the position of a common intersection point, if this exists, of a family of l lines, each of them determined by m points (Fig. 3a). In our case all substrates were studied at the same set of temperatures. According to this method a constraint of a common intersection point is made, and the residual sum of squares S_0 with $f = (m - 1)l - 2$ degrees of freedom is calculated. This sum is compared to the sum of squares S_{00} with $f = (m - 2)l$ degrees of freedom, calculated from the regression lines without the constraint of a common point of intersection. After applying Exner's treatment (the sixth compound was eliminated) we obtained $S_0 = 0.07502$ with $f = 46$ and $S_{00} = 0.07295$ with $f = 42$. The corresponding standard deviations are $s_0 = 0.040$ and $s_{00} = 0.042$ log units, respectively. Since $s_0 < s_{00}$ the isokinetic hypothesis is valid. The isokinetic temperature calculated by this method is 472°K. In order to obtain the confidence interval of β, the standard deviation as a function of the supposed

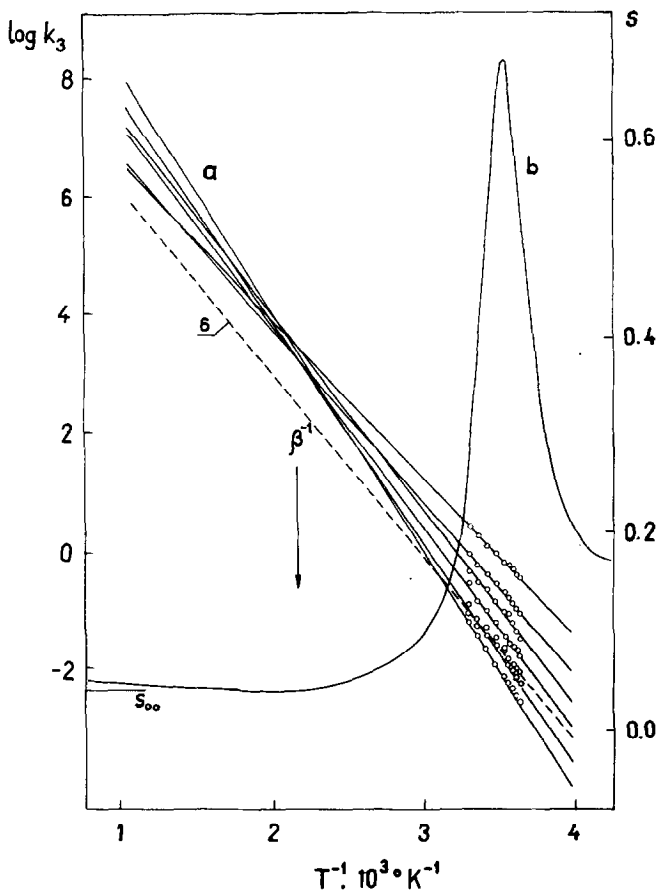

FIG. 3. Data treatment according to Exner (3). (a) Arrhenius plot; (b) the standard deviation, s, as a function of supposed isokinetic temperature. (The sixth compound is eliminated.)

isokinetic temperature was plotted (Fig. 3b). The minimum of this curve was very flat; so the isokinetic temperature could not be given any distinct value, though the compensation effect was considered proved.

Recently Krug *et al.* (*4*) proposed a method which distinguishes between the statistical and the chemical compensation effect. A statistical test based on the null hypothesis

$$H_0\colon \beta = T_{hm}$$

may be used to discriminate between the two patterns. We have analyzed our data by the method suggested by Krug *et al.*, and for the $\Delta H^{\neq} - \Delta S^{\neq}$ plot we obtained an isokinetic temperature of 454 ± 43°K (P = 95%). The harmonic mean of our experimental temperatures T_{hm} = 286°K does not fall in this interval. Therefore the null hypothesis can be rejected, and we can assert that the observed enthalpy–entropy compensation pattern has a chemical causation.

In order to estimate β with higher precision as proposed by Krug *et al.* (*22*) we

used the following equivalent formulation of the Arrhenius equation:

$$\ln k = \{\ln A - E/RT_{hm}\} - E/R\{1/T - 1/T_{hm}\}.$$

The activation free energy, estimated from the intercept is not correlated with the enthalpy of activation derived from the slope (the correlation parameter $\rho_{\Delta H^{\neq},\Delta G^{\neq}_{T_{hm}}} = 0$). A linear relationship in $\Delta H^{\neq} - \Delta G^{\neq}_{T_{hm}}$ plane with a slope γ is a reflection of a linear relationship in $\Delta H^{\neq} - \Delta S^{\neq}$. The slope γ is related to the isokinetic temperature by the equation:

$$\beta = T_{hm}/(1 - 1/\gamma).$$

The linear dependence obtained is shown on Fig. 4. By the use of the ordinary least-squares linear regression after excluding the sixth compound, $\gamma = 2.537$ ($r = 0.993$), and $\beta = 472°K$ were calculated.

M-regression procedure with error in both variables gave $\gamma = 2.660$ with its 95% confidence interval ($\gamma_L = 2.062$, $\gamma_U = 3.258$) and $\beta = 458°K$ ($\beta_L = 412°K$, $\beta_U = 555°K$).

The β values obtained by all methods agree well (see Table 2).

In 1947 Hinshelwood characterized the compensation effect as one of the essential phenomena in the chemical kinetics "which are still mysterious" (*23*). The enthalpy–entropy compensation is observed in a great number of investigations, but there is no general theory of this phenomenon up to now. The compensation effect is assumed to be an indication of a single mechanism for the whole reaction series, i.e., that all reactions pass through the same rate-limiting elementary step (*24*).

The formation of the hydrophobic contact between two appolar groups in aqueous solution is accompanied by positive enthalpy and entropy changes (*25*). In the reaction series we studied, the increase of the catalytic constant of the

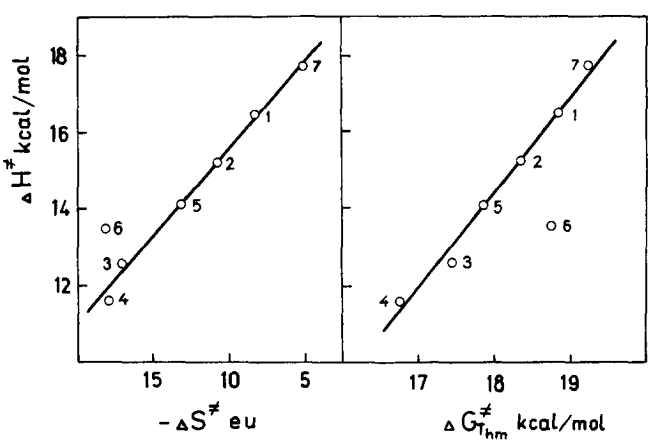

FIG. 4. (a) $\Delta H^{\neq}/\Delta S^{\neq}$ and (b) $\Delta H^{\neq}/\Delta G^{\neq}$ plots of thermodynamic parameter estimates for the hydrolysis of *p*-nitrophenyl carboxylates catalyzed by alkaline mesentericopeptidase. The data are evaluated at $T_{hm} = 286°K$. (The indication of the points corresponds to *n*. The sixth compound is eliminated.)

TABLE 2

ISOKINETIC TEMPERATURE VALUES OBTAINED BY MEANS OF DIFFERENT METHODS FOR THE DEACYLATION STEP OF THE HYDROLYSIS OF *p*-NITROPHENYL CARBOXYLATES CATALYZED BY ALKALINE MESENTERICOPEPTIDASE

Method	β (°K)	95% Confidence Interval of β
Exner (19)	490	(343, 637)
Petersen (21)	500	
Exner (3)	472	
Krug (4): null hypothesis	454	(411, 497)
Krug (22): ordinary linear regression	472	(437, 533)
Krug (22): *M*-linear regression	458	(412, 555)

substrates leads to a decrease of both the enthalpy and entropy of activation. According to (*26*) this can be interpreted as due to breaking of the hydrophobic contacts upon passing from the ground to the transition state of the deacylation step, and the hydrophobic side chain in part, or completely leaves the hydrophobic cavity. This process may induce the formation of new bonds, which leads to a negative enthalpy change, restriction of the molecular motion, resulting in enthalpy–entropy compensation.

According to Lumry and Rajender (*1*) the compensation effect may be used as a diagnostic test for the participation of water in protein processes. It was suggested that conformational changes are accompanied by disordering of the water clusters formed over the protein surface (*1, 27*). This process is identified as melting of ice, which is a first-order phase transition with

$$\Delta G = 0 \quad \text{and} \quad \frac{\Delta H^{H_2O}}{\Delta S^{H_2O}} = 273°\text{K}.$$

The nonpolar regions are adapted to water in such a manner that these processes can occur at higher temperature. Lumry claims that the value of the isokinetic temperature, β, of nearly 300°K is of particular importance and may be considered as a means of the thermostated organisms to keep independent of their surroundings by maintaining constant the free energy of activation by $\Delta H/\Delta S$ compensation. Subtle-change processes including both protein geometry and solvent state in these organisms occur with small free energy change, which is possible only near their compensation temperature (*28*).

The isokinetic temperature values we obtained by means of various treatments are significantly higher than 300°K (Table 2). Similar higher values have been reported for the deacylation step of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl carboxylates: 435°K (*29*) and 420°K (*6*); we obtained β values of 427°K (without the octanoate) and 450°K after calculation of the data in (*7*) and (*30*). In α-chymotryptic hydrolysis of other series, β values of 400–450°K were obtained for the acylation (*31*) and the deacylation (*6, 26*) step. Data from recent

thermodynamic investigations of the interaction of water with α-chymotrypsin (*32*) and the association of Bowman–Birk inhibitor with α-chymotrypsin (*33*) also show the isokinetic temperature to be about 400°K. These results suggest that water is not the unique source of the enthalpy–entropy compensation in enzyme reactions.

The present study is the first one carried out with a proteinase of bacterial origin. The results are in good agreement with those reported for mammalian proteinases and imply a common mechanism of action of the enzymes derived from mammalian and bacterial species.

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