General method for exact evaluation of parameters of the elementary steps of coupled reactions

Invariant analysis

Bent H. HAVSTEEN

Biochemisches Institut der Medizinischen Fakultät, Christian-Albrechts-Universität zu Kiel, Olshausenstrasse 40, D-2300 Kiel, Federal Republic of Germany

A general method has been devised for the exact evaluation of the rate constants of the elementary steps of a system consisting of any number of coupled reactions. The method is independent of the structure of the network of coupled steps. The precision of the data evaluation is solely dependent on the quality of the detection unit. The application of the method is illustrated with data collected for the binding of the competitive inhibitor proflavine to chymotrypsin under such conditions that five states of the enzyme are required to interpret the results. In the absence of a substance possessing the binding specificity, the enzyme is present as an equilibrium between an active and an inactive conformer. The latter prevails. The binding of the specific inhibitor releases a slow proton transfer from the medium to the α -amino group of Ile-16. Subsequently, the enzyme-inhibitor (or enzyme-substrate) complex re-arranges to the catalytically active form, which is retained until the supply of specific substrate is exhausted. The control features described are general, but are particularly conspicuous under the special environmental conditions used here. A comparison between data for α - and δ -forms of chymotrypsin showed that the chain ends of the former impeded the substrate binding and that the activity controlling conformational change occurred in the interior of the enzyme molecule.

INTRODUCTION

Chemistry abounds with examples of coupled reactions. Many of these reaction systems are very complex. They are therefore often described by overall parameters. The development of the chemical relaxation techniques by Eigen and his co-workers has permitted the elucidation of mechanisms of a complexity that previously rendered them intractable, especially if they contained very rapid and many strongly coupled steps [1]. However, although the arithmetic relationships between the relaxation times observed and the concentrations of the reaction species involved could often be formulated, great difficulties were encountered by the manipulation of the very complex expressions that often emanated. Therefore such expressions were usually truncated or overall parameters were resorted to [2]. The resulting loss of information may be avoided by an analysis of the invariants of the system.

THEORY

Most biochemical processes, e.g. all enzyme mechanisms, consist of a considerable number of steps, which are coupled in a network that in many ways resembles electronic circuitry. However, in contrast with the electrical analogy, the particles flowing are not necessarily electrons but include a number of chemical substances, which often are structurally related. If such a system at equilibrium is disturbed by a change in an environmental parameter, e.g. the temperature or the pressure, that is rapid compared with the chemical reactions, and if at least one of the steps is accompanied by a significant

change in the associated intensive thermodynamic function, in this case the enthalpy, ΔH , or the molar volume, ΔV , then the network will respond by a surge of substances that flow through it in such directions that a new equilibrium state, specified by the new environmental conditions, is approached. If the network consists of n connected states, it will respond with $n-1$ chemical relaxation effects, i.e. approaches to new local equilibria. These relaxation effects may be monitored by using the accompanying changes in optical absorption, fluorescence, optical rotation, electrical conductivity or any other convenient physical property.

If the perturbation is small, e.g. a few degrees, then the relaxation effects will be simple concentration-dependent exponential functions in the time elapsing. Their time constants, the relaxation times, τ_i , may be evaluated from semi-logarithmic plots. These τ values do not pertain directly to single steps, but are normal modes of a group of elementary reactions. However, the rate constants and other properties of individual steps may be derived from the concentration-dependencies of the relaxation times as follows.

When a node in the reaction network, a state connected to other states through elementary reactions, experiences a sudden perturbation, such as a temperature change, then the equilibrium constants of the associated steps simultaneously change according to Van't Hoff's law ($\ln K = -\Delta H/RT + \Delta S/R$). The resulting flow of substances to and from the perturbed state is a linear combination of the rate constants and the concentrations of the species involved. Since the system is restricted by a conservation law, the sum of all concentration

perturbations is zero and the n perturbed states characterized by only $(n-1)$ such linear combinations or rate equations, i.e. by $(n-1)$ relaxation effects:

$$\begin{aligned} dx_1/dt = \dot{x}_1 &= a_{11}x_1 + a_{12}x_2 + \dots \\ \dot{x}_2 &= a_{21}x_1 + a_{22}x_2 + \dots \\ &\vdots \\ &\vdots \end{aligned} \quad (1)$$

where a_{ij} are functions of the rate constants of elementary steps and the concentration species whereas x_i is the concentration of a representative species in the state i [1]. The equations became linear since the concentration perturbations are so small (approx. 1%) that terms containing powers of x greater than 1 may be ignored.

Such a set of $(n-1)$ simultaneous equations is formally analogous to those of a set of vectors in $(n-1)$ dimensions with x_i as unit vectors and a_{ij} as co-ordinate values. These $(n-1)$ rate equations together characterize the response of the chemical reaction network to the perturbation. The $\dot{x}_i$ s are the eigenvectors of the resultant vector or its equivalent in the reaction network analogy.

Accordingly:

$$\begin{aligned} a_{11}x_1 + a_{12}x_2 + \dots &= \lambda x_1 \\ a_{21}x_1 + a_{22}x_2 + \dots &= \lambda x_2 \\ &\vdots \\ &\vdots \end{aligned}$$

or

$$\begin{aligned} (a_{11} - \lambda)x_1 + a_{12}x_2 + \dots &= 0 \\ a_{21}x_1 + (a_{22} - \lambda)x_2 + \dots &= 0 \\ &\vdots \\ &\vdots \end{aligned} \quad (2)$$

where $\lambda = 1/\tau$ = the eigenvalues of the system and τ is one of the $(n-1)$ relaxation times.

Such a system of simultaneous equations may be solved by the determinantal method. A matrix A is constructed from the coefficients of x_i yielding the characteristic equation:

$$(A - \lambda E) = \begin{vmatrix} (a_{11} - \lambda) & a_{12} & \dots & a_{1(n-1)} \\ a_{21} & (a_{22} - \lambda) & \dots & a_{2(n-1)} \\ \vdots & \vdots & \ddots & \vdots \\ a_{(n-1)1} & \dots & \dots & (a_{(n-1)(n-1)} - \lambda) \end{vmatrix} = 0 \quad (3)$$

where E is the unit matrix.

This matrix is developed to give a characteristic polynomial (eqn. 4) in the $(n-1)$ th degree in λ and with the invariants as coefficients. The invariants are properties of the vectorial system that remain unchanged under a linear change in co-ordinates or a rotation of the co-ordinate system. In the chemical-network analogy, the invariants are intrinsic properties of this structure that are not significantly affected by minor perturbations in environmental parameters, such as those used experimentally.

The characteristic polynomial is:

$$(-\lambda)^{n-1} + I_1(-\lambda)^{n-2} + I_2(-\lambda)^{n-3} + \dots + I_{n-2}(-\lambda) + I_{n-1} = 0 \quad (4)$$

where I_i is the i th invariant (see below).

Previously, the characteristic polynomial (eqn. 4) has almost invariably been solved numerically in spite of inherent ambiguities or truncated with the resulting loss

of information. However, such approximations are unnecessary, since the invariants are simple linear functions in the rate constants and the concentrations, and their number is always sufficient to permit a mathematically exact evaluation of all the rate constants of the elementary steps in the reaction network. The reason is that, if there are n elementary steps, i.e. $2n$ rate constants to be found, then the associated invariants plus the mass conservation rules, e.g. for enzyme and substrate, plus the two Kirchhoff laws always yield a number of linear equations in the rate constants that is not less than $2n$. Each invariant has a concentration-dependent term and a concentration-independent term, thus yielding $2n$ equations in the rate constants. Both of Kirchhoff's laws, which were developed for electrical circuits, are equally valid to a ring of chemical reactions. One of them states that the product of all rate constants around the ring in one direction is equal to the product of the rate constants in the other direction, i.e. $\prod K_i = 1$ where the K_i s are equilibrium constants.

A curiosity arising from Kirchhoff's laws applied to reaction networks is that the sum of each column in eqn. (3) is zero.

An example of a model is seen in Fig. 1. The corners represent the states. One example is state ①, which contains $E_a + S + H^+$. The edges of the model represent a pair of elementary reactions. An example is the reaction from state ① to state ②:

which represents the interconversion between the active and the inactive form of the enzyme through a conformational change. This step is characterized by the rate constants k_{12} and k_{21} .

If the rate constants of the elementary steps have been estimated by other means other than by the use of chemical-relaxation methods, the consistency of the model can be tested by invariant analysis. In that case, the relaxation times may be computed from the rate constants and the concentrations by using the equations given in previous papers.

Fig. 1. Model of the reaction states and their interconversions during the interaction of α -chymotrypsin (E) with a specific substrate or inhibitor (S)

The indices a and i refer to the active and inactive states of the enzyme. The major reaction pathway is indicated by the heavy line.

The Invariants [3]

$$I_1 = \sum_{i=1}^{n-1} \lambda_i = \sum_{i=1}^{n-1} a_{ii}$$

= the sum of the diagonal terms
in the matrix A

(5)

$$I_2 = \sum_{i>j, j=1}^{n-1} (\lambda_i \lambda_j) = \sum_{i>j, j=1}^{n-1} A \begin{pmatrix} ij \\ ij \end{pmatrix}$$

$$= \sum_{i>j, j=1}^{n-1} (a_{ii}a_{jj} - a_{ij}a_{ji})$$
(6)

$$I_3 = \sum_{i>j>k, k=1}^{n-1} (\lambda_i \lambda_j \lambda_k) = \sum_{i>j>k, k=1}^{n-1} A \begin{pmatrix} ijk \\ ijk \end{pmatrix}$$

$$= - \sum_{i>j>k, k=1}^{n-1} {}^3D$$
(7)

$$I_p = \sum_{i>j>k \dots >p, p=1}^{n-1} (\lambda_i \lambda_j \lambda_k \dots \lambda_p)$$

$$= \sum_{i>j>k \dots >p, p=1}^{n-1} A \begin{pmatrix} ijk \dots p \\ ijk \dots p \end{pmatrix}$$
(8)

$$I_{n-1} = \prod_{i=1}^{n-1} \lambda_i = \text{the scalar value of } A$$
(9)

where 3D is a third-order minor with elements carrying the indices of D as diagonal elements. The indices i, j, k etc. are circularly permuted, each number occurring only once and $i \neq j \neq k$ etc.

Example: $n = 4$

$$I_3 = D_{112233} + D_{223344} + D_{334411} + D_{441122}$$

$$D_{223344} = \begin{vmatrix} a_{22} & a_{23} & a_{24} \\ a_{32} & a_{33} & a_{34} \\ a_{42} & a_{43} & a_{44} \end{vmatrix}$$

The subsequent invariants may be constructed similarly (see Table 1) [4].

Example of the application of invariant analysis

About 20 years ago, a transition of chymotrypsin and its specific acyl-enzymes was observed at slightly alkaline pH, 15–20 °C and physiological ionic strength [5]. Since difficulties were encountered in fitting the data collected on the binding interactions of chymotrypsin with its specific inhibitor proflavine under such conditions to simple models, the possibility was considered that a

pre-equilibrium between two conformers of the enzyme was contributing to the mechanism. The most simple assumption is that this conformational change is essentially the same as the one that has been described for the enzyme-inhibitor complex. In that case the pre-equilibrium represents an activation-inactivation rearrangement (see Fig. 1). This system was investigated by invariant analysis.

MATERIALS AND METHODS

Bovine δ -chymotrypsin was purchased from Sigma Chemical Co. and proflavine hemisulphate from Fluka. The purity and activity were checked as previously described. Both substances were dissolved in 50 mM-sodium phosphate buffer adjusted to pH 8.3. An equilibrium mixture of the enzyme and its competitive inhibitor (a substrate analogue) were in a vessel under temperature control subjected to temperature jumps of 4.4 °C to a final temperature of 17.5 °C in an apparatus purchased from Messanlagen, Göttingen, Germany. The course of the reactions released by the temperature jump was followed by recording of the absorption at 475 nm as a function of time. The chemical relaxation times that occurred in the range 30 μ s–50 ms were evaluated from semi-logarithmic plots. The equilibrium constants used to calculate the equilibrium concentrations of various species were estimated from previous data [6].

RESULTS AND DISCUSSION**Model**

The model used is shown in Fig. 1. The enzyme is present in two conformations, an active one, E_a , and an inactive one, E_i [6–9]. Their interconversion is controlled by a proton transfer, probably between water and the α -amino group of Ile-16. The active form of the enzyme is assumed to be the protonated one because it favours the formation of the Ile-16–Asp-194 salt linkage, which is known to be associated with activity. The binding of both the proton and the specific substrate or inhibitor is assumed to promote the activation process [10], since the converse would be disadvantageous to the organism and would be expected to be eliminated by evolution. In the simple version of the model, only five of the 12 states that it contains are assumed to be significantly populated,

Table 1. Characteristic invariant polynomials in the eigenvalues, λ_i , for coefficient matrices of the order n

The indices i, j, k etc. are circularly permuted, each number occurring only once and $i \neq j \neq k$ etc.

n	Polynomial	
2	$\lambda^2 - \lambda \sum_{i=1}^2 a_{ii} + \sum_{i>j, j=1}^2 (a_{ii}a_{jj} - a_{ij}a_{ji})$	$= 0$
3	$\lambda^3 - \lambda^2 \sum_{i=1}^3 a_{ii} + \lambda \sum_{i>j, j=1}^3 (a_{ij}a_{jj} - a_{ij}a_{ji}) - {}^3D$	$= 0$
4	$\lambda^4 - \lambda^3 \sum_{i=1}^4 a_{ii} + \lambda^2 \sum_{i>j, j=1}^4 (a_{ii}a_{jj} - a_{ij}a_{ji}) - \lambda \sum {}^3D_{ii, jj, kk} + {}^4D$	$= 0$
$\vdots$		
n	$\lambda^n - \lambda^{n-1} \sum_{i=1}^n a_{ii} + \lambda^{n-2} \sum_{i>j, j=1}^n (a_{ii}a_{jj} - a_{ij}a_{ji}) - \lambda^{n-3} \sum {}^3D_{ii, jj, kk} + \dots + (-1)^n D^n$	$= 0$

Fig. 2. Dependence of the reciprocal of the relaxation times on the sum of the equilibrium concentrations of the inhibitor and the enzyme in the inactive form

The curves represent theoretical values computed from the rate constants in Table 1 (five states). The broken portions of the curves represent a concentration range in which an exact evaluation was hampered by the experimental error. The very slow relaxation effect was very difficult to measure because of its low amplitude. ○, ●, □ and × symbols represent observed relaxation times.

because that is sufficient to satisfy the observations at the present signal/noise ratio (see Fig. 2). Also, four states, labelled ②–⑤ inclusive, satisfy the data quite well, which indicates that the enzyme in the absence of a substance possessing the binding specificity is mainly present in the inactive form (see Fig. 3). This situation is advantageous because it prevents a blockade of the catalytic site with unspecific substances.

Population of the states

Specific inhibitors of chymotrypsin in the alkaline range drive this enzyme into a conformation which is different from the one that predominates in the absence of such ligands, i.e. substances carrying the specificity determinant shift the pre-equilibrium of enzyme conformers. All available evidence suggests that only two conformers of the enzyme need to be considered [10]. Chymotrypsin that is specifically acetylated at the amino group of Ile-16 is enzymically inactive. Nevertheless it is capable of rearranging into the alternative conformer. Since the ionic linkage between Asp-194 and Ile-16 does not exist in specifically monoacetylated δ -chymotrypsin, the con-

formational change detected must be the tightening-relaxation isomerism in the vicinity of the specific binding site for substrate and Ile-16.

The conformation into which indole, and presumably also proflavine, above pH 8 drives the enzyme is, according to Garel & Labouesse [10], the active one, E_a . This concept agrees well with my data. When the enzyme is fully saturated with a specifically binding substance, only one enzyme conformer is discernible, as judged by the polarimetric parameters. Its stability is in the physiological range independent of pH. the structure of this conformer is indistinguishable from that of E_aH^+ . Hence the states ②–④ inclusive are expected to predominate at high concentrations of the specific ligand. Although the concentrations of E_i and E_iH^+ are vanishing at neutral pH, both of these two enzyme conformers, E_i and E_a , are present in the pH range of slight alkalinity and their equilibrium is shifted towards the E_a form by the binding of a specific substance or the proton. In the alkaline pH range the proton uptake by the enzyme is only possible when the recognition by the binding site of a specific substance has led to a pK change of the Ile-16 α -amino group. Therefore the binding order of the two ligands is obligatory: substrate or its analogue before the proton. Hence the states ①–⑤ inclusive must be those of the eight states of the models that must be assumed to be the more populated and that primarily must be considered in the kinetic analysis. This expectation was confirmed by the kinetic data.

Evaluation of the rate constants

The invariants were computed for each model tested from the measured relaxation times by using the equations given in the Theory section. Each invariant was plotted as a function of a concentration term as shown in Figs. 2–4 and explained above. The slope and ordinate intercept defined by each invariant line yielded a number of equations in the rate constants equal to the number of rate constants, thus permitting an exact evaluation of the latter. The experimental errors incurred by this method were evaluated from the variances of the observations from the regressed invariant lines. The parameters from the invariants (Fig. 4 and Table 2) were used to construct the theoretical curves, which agree with the observations within the experimental error. The slow effect is difficult to detect because its amplitude is very small. The magnitude of the rate constants determined agree with the expectations from other systems, with the exception of the proton transfer, which is very slow in the direction of dissociation. The rate of association of the specific inhibitor with the enzyme is almost diffusion-controlled. This was also found by the binding of other small substances to proteins [11]. The rates of the conformational changes also lie in the usual range. The two binding processes in this case favour the conversion of the enzyme into the inactive form ($k_{21}/k_{12} = 2.3 \pm 0.5$, $k_{45}/k_{54} = 0.7 \pm 0.2$). Similar equilibrium data have previously been found [10].

Slow proton dissociation from Ile-16 locks the active conformation

Most proton transfers, both association and dissociation reactions, occur very rapidly, unless special conditions, e.g. steric hindrance, unfavourable electric fields or a low local dielectric constant, prevail [1]. Since substances that are specific for chymotrypsin carry a

Fig. 3. Evaluation of data from about 100 temperature-jump experiments on the α -chymotrypsin/proflavine system at pH 8.3 and 17.5 °C (final temperature) in 50 mM-phosphate buffer by using the four-state model

(a) Concentration-dependence of the invariant $I_1 = -(1/\tau_1 + 1/\tau_2 + 1/\tau_3)$. (b) Concentration-dependence of $I_2 = (1/\tau_1)(1/\tau_2) + (1/\tau_2)(1/\tau_3) + (1/\tau_3)(1/\tau_1)$. (c) Concentration-dependence of $I_3 = -(1/\tau_1)(1/\tau_2)(1/\tau_3)$. The points shown are average values. The deviations from linearity show the limitations of this model.

Fig. 4. Invariants pertaining to the model that contains the states

②–⑤ inclusive in Fig. 1

$$k'_{23} = k_{23}([E] + [S]).$$

hydrophobic determinant, and since the binding of such substances are known to displace three water molecules from the binding site, a low polarity would be expected to exist in the vicinity of the α -amino group of Ile-16 that switches the enzymic activity on [1, 12–14]. Despite the small size of water (or the proton), its rate of penetration to the α -amino group would therefore be expected to be low. However, the rate constant found was quite normal for such a process. After formation of the Ile-16–Asp-194 salt linkage, the specific binding zone including the *N*-terminus of the B-chain (Ile-16) is known to contract into a denser configuration [14]. Hence the return of the proton to the medium would also be expected to occur very slowly, which was in fact found (Table 1). Since the magnitude of the amplitude of the slow relaxation effect is related to and, in this case of an almost uncoupled step, dominated by the relative displacement of the binding constant caused by the temperature jump, this displacement must, under the existing special conditions, be small, i.e. the associated enthalpy of ionization is low. When an α -amino group is in contact with water, its ΔH_i value is above about 42 kJ/mol (10 kcal/mol), but in the present case it must be much smaller [15]. Since little is known about the factors that influence the heat of ionization, it is not possible to give this phenomenon a definite explanation. However, it is known that anions such as carboxylate and phosphate have a low enthalpy of ionization and that cations containing N have a large ΔH_i value. Therefore the structure of water near the ions is probably playing an important role [16]. In the present case water is scarce at the site in question and therefore the processes that are expected to be responsible for the enthalpy change are likely to be almost absent. The slow

Table 2. Comparison between the parameters derived from different models

Rate constant	Three states		Four states		Five states		Weighted average
	Exact evaluation*§	Evaluation based on $K_E^\dagger$	Exact evaluation†	Approximate evaluation¶	Exact evaluation		
k_{12} (s ⁻¹)	—	—	—	1612 ± 830	1115 ± 210	1248 ± 340	
k_{21} (s ⁻¹)	—	—	—	2042 ± 390	3288 ± 300	2885 ± 310	
k_{23} (M ⁻¹ ·s ⁻¹)	$(1.66 \pm 0.08) \times 10^8$	$(3.44 \pm 0.03) \times 10^8$	$(4.9 \pm 0.2) \times 10^8$	$(5.8 \pm 0.2) \times 10^8$	$(1.84 \pm 0.05) \times 10^9$	$(6.2 \pm 1) \times 10^8$	
k_{32} (s ⁻¹)	523 ± 30	1444 ± 310	1913 ± 130	≤ 200	≤ 200	1189 ± 90	
k_{34} (M ⁻¹ ·s ⁻¹)	*	$(5 \pm 1) \times 10^{10}$	$(1.57 \pm 0.08) \times 10^9$	$(8 \pm 5) \times 10^{10}$	$(1.57 \pm 0.08) \times 10^9$	$(9.6 \pm 1.6) \times 10^9$	
k_{43} (s ⁻¹)	*	250 ± 50	≤ 160	≤ 160	190 ± 130	237 ± 90	
k_{45} (s ⁻¹)	3026 ± 670	1857 ± 880	1367 ± 90	5440 ± 1100	1183 ± 750**	2754 ± 470	
k_{54} (s ⁻¹)	251 ± 150	5570 ± 2350	4100 ± 560	≤ 3000	3547 ± 2250**	3804 ± 930	
$k_{45} + k_{54}$ (s ⁻¹)	3277 ± 690	7427 ± 2510	5467 ± 590	5440 ± 3190	4730 ± 2370	5169 ± 900	

* The proton-transfer step is assumed to be uncoupled from the remainder of the steps.

† The equilibrium concentration of EI is taken into account by the calculation of the equilibrium concentration of the other species, although the parameters were derived at $c \rightarrow 0$ where EI is absent.

‡ $K_E = 6.67 \times 10^{-5} \text{ M}^{-1}$ was obtained by interpolation of data from other conditions.

§ EI was not taken into account in the calculation of the equilibrium concentration of other species for the reason mentioned in footnote †.

$$\| \bar{x} = \sum_{i=1}^n (x_i/s_{x_i}) / \left(\sum_{i=1}^n s_{x_i} \right)^{-1}.$$

¶ Based on inequalities defining the conditions for real values of the parameters.

** Only obtainable assuming $k_{45}/k_{54} = 0.33$ (from the four-state model) since the experimental errors are too large for a direct evaluation.

Table 3. Comparison between the kinetic parameters of α - and δ -forms of chymotrypsin at pH 8.3

Enzyme	k_{12} (s ⁻¹)	k_{21} (s ⁻¹)	$10^{-8} \times k_{23}$ (M ⁻¹ ·s ⁻¹)	k_{32} (s ⁻¹)	Temperature (°C)
α -Form*	800 ± 400	5000 ± 1000	1.0 ± 0.4	1200 ± 400	12.0
δ -Form	1250 ± 350	2900 ± 300	6 ± 1	1200 ± 90	17.5

* Ref. [5].

proton transfer delays the onset of the catalytic effect. This was also observed as a pre-steady-state phase in the chymotryptic cleavage of *N*-acetyl-L-tyrosine ethyl ester under the same environmental conditions.

Once the enzyme has assumed its active conformation, this structure is locked as a consequence of the slow rate constant of the reversal of the re-arrangement, which in turn is due to the electrostatic stabilization of the α -ammonium ion by Asp-194. The active conformation is retained until its supply of specific substrate is exhausted. These properties are not restricted to the special environmental conditions used here, but the latter enhance the controlling features, thus facilitating their analysis.

Comparison between the kinetic parameters of α - and δ -forms of chymotrypsin at pH 8.3

When chymotrypsinogen, which only consists of a single peptide chain, is activated, the first proteolytic cleavage produces an active enzyme, π -chymotrypsin, which rapidly is converted into the δ -form by release of a dipeptide from the new chain end. Therefore the latter

enzyme consists of two peptide chains, A and B, which are connected by disulphide bonds. The α -form arises from δ -chymotrypsin by autocatalytic liberation of a second dipeptide from the interior of the B-chain. Therefore α -chymotrypsin consists of three peptide chains, A, B and C, which are covalently cross-linked by disulphide bonds.

The kinetic parameters of α - and δ -forms of chymotrypsin are compared in Table 3. Although the experimental temperature was somewhat different, the rate constant for the association of proflavine to δ -chymotrypsin was markedly larger than that for α -chymotrypsin, because the low activation energy causes only a small error. In contrast, the rate constants for the dissociation of the ligand from the two enzymes were identical. Apparently the C-chain of α -chymotrypsin impedes the association of the ligand. The rate constants for the conformational changes of the two enzymes hardly differ when the difference in temperature that here has a significant influence is considered. This result supports the hypothesis that the conformational change occurs in the interior of the enzyme and is hardly afflicted by the chain ends.

REFERENCES

1. Eigen, M. & DeMayer, L. (1963) in *Techniques of Organic Chemistry*, vol. 8 (Weissberger, A., ed.), pp. 895–1054, Interscience, New York
2. Schwarz, G. (1968) *Rev. Mod. Phys.* **40**, 206–218
3. Madelung, E. (1964) *Die Mathematischen Hilfsmittel des Physikers*, 7th edn., pp. 206–207, Springer-Verlag, Berlin
4. Turnbull, H. W. (1960) *The Theory of Determinants, Matrices and Invariants*, pp. 132–138, Dover Publications, New York
5. Havsteen, B. (1968) Ph.D. Dissertation, University of Copenhagen
6. Havsteen, B. (1967) *J. Biol. Chem.* **242**, 769–771
7. Fersht, A. R. (1972) *J. Mol. Biol.* **64**, 497–509
8. Taylor, R. P., Vatz, J. B. & Lumry, R. (1973) *Biochemistry* **12**, 2933–2940
9. Quast, V., Engel, J., Heumann, H., Krause, G. & Steffen, E. (1974) **13**, 2512–2520
10. Garel, J.-R. & Labouesse, B. (1970) *J. Mol. Biol.* **47**, 41–56
11. Pecht, I. (1983) in *Protein Conformation as an Immunological Signal* (Celada, F., Schumaker, V. N. & Sercarz, E. E., eds.), pp. 3–13, Plenum Press, New York
12. Böning, W. & Havsteen, B. (1981) *J. Biol. Chem.* **257**, 6836–6843
13. Hess, G. P., McConn, J., Ku, E. & McConkey, G. (1970) *Philos. Trans. R. Soc. London Ser. B* **257**, 89–104
14. Blow, D. M. (1976) *Acc. Chem. Res.* **9**, 145–152
15. Edsall, J. T. & Wyman, J. (1958) *Biophysical Chemistry* p. 533, Academic Press, New York
16. Havsteen, B. (1984) *J. Biophys. Chem.* **20**, 305–317

Received 21 February 1985/8 July 1985; accepted 13 August 1985