Journal of Solid-Phase Biochemistry, Vol. 5, No. 2, 1980

THERMAL INACTIVATION OF IMMOBILIZED ENZYMES: A KINETIC STUDY

JENS FISCHER, RENATE ULBRICH, ROMANA ZIEMANN, SABINE FLATAU, PETER WOLNA, MARTIN SCHLEIFF, VOLKER PLUSCHKE, and ALFRED SCHELLENBERGER

Institute of Enzymology and Technical Microbiology, DDR-1017 Berlin
Section of Biosciences and Section of Mathematics
Martin-Luther University, DDR-402 Halle, G.D.R.

Accepted December 21, 1979

The thermal inactivation kinetics of chymotrypsin, trypsin, and α-amylase bound to silica, polyacrylamide, and polystyrene were studied at different temperatures. The inactivation curves were analyzed by a kinetic model, assuming a first-order reaction of differently stable enzyme fractions on the matrix. In all cases the assumption of two enzyme fractions with distinctly different inactivation constants was sufficient for describing the inactivation progress (standard deviations between experimental and calculated inactivation curves, 1–4%). Both the inactivation constants as well as the relative concentrations of the enzyme fractions were found to change in dependence on temperature.

INTRODUCTION

The application of immobilized enzymes in analysis, medicine, and technology has raised the general interest in stable (including thermostable) enzymes.[1] The thermostability of immobilized enzymes was found to be higher, similar, or lower than that of the corresponding soluble enzymes (1). Multipoint attachment of an enzyme to a matrix could be shown to be one principle of enzyme stabilization (2–4). Usually the thermostability is studied by heating enzyme samples and following their activity decrease in dependence on heating time and temperature. An alternative method is differential scanning calorimetry (3,5), which indicates enthalpy changes on heating an enzyme sample.

The results of several publications reveal that the thermal inactivation of immobilized enzymes, in contrast to most soluble enzymes, does not obey

[1] Abbreviations used in this paper. TAP-silica-chymotrypsin (trypsin): chymotrypsin (trypsin) bound to s-trichlorotriazine-activated γ-aminopropyl silica; SuAP-silica-chymotrypsin (trypsin): chymotrypsin (trypsin) bound to γ-succinamidopropyl silica; PA-chymotrypsin: polyacrylamide-bound chymotrypsin; QPS-amylase: α-amylase bound to 1,4-benzoquinone-activated aminomethyl polystyrene.

0146-0641/80/0600-0079 $03.00/0

first-order kinetics (4,6–8). These findings can be explained assuming that the immobilization results in several populations of bound enzyme molecules, which differ in mode and number of covalent bonds as well as in noncovalent protein-matrix interactions. Nonidentical linkages and interactions between protein and matrix are above all caused by steric differences in the polymer structure (9–11). Since mode and number of linkages and interactions affect the conformational stability of the bound enzyme molecules, there can be molecules with different stability in one derivative. To describe the kinetics of thermoinactivation in immobilized enzymes quantitatively, Martinek et al. (4) used "effective" first-order rate constants, and Cardoso and Emery (8) determined rate constants from a linear decay model.

In the present work, we report on a kinetic model describing the thermal inactivation of immobilized enzymes by first-order kinetics (12). Assuming that the immobilization results in differently stable populations of bound enzyme molecules, the total inactivation process is described by

$$A_t/A_0 = \sum_i x_i \exp(-k_i t) \tag{1}$$

where A_t is the enzyme activity after the heating time t, A_0 is the activity before heating, k_i represents the rate constant of inactivation, and x_i is the relative concentration of the enzyme fraction $i(\sum_i x_i = 1)$. For testing the model, we have studied the thermal inactivation of chymotrypsin, trypsin, and α-amylase, which were immobilized on porous silica, polyacrylamide, and polystyrene by several covalent binding methods.

MATERIALS AND METHODS

α-Chymotrypsin was obtained from Boehringer, Mannheim; trypsin was purchased from Spofa, Prag. Fungal α-amylase (*A. oryzae*) was a commercial product of Novo Industri A/S, which was purified by gel filtration on Sephadex G100. The content of active sites in chymotrypsin (89%) and trypsin (59%) was determined by 4-nitrophenyl acetate (13) and 4-nitrophenyl-4'-guanidine-benzoate·HCl (14), respectively.

Porous silica (particle size, 200–400 μm; pore volume, 0.96 cm^3/g; average pore diameter, 200 Å) was a gift from the Department of Technical Chemistry, Martin-Luther University, Halle. Cross-linked polyacrylamide containing hydazide groups (Enzacryl AH) was from Koch-Light Laboratories, England. Wofatit Y58 (aminomethyl) polystyrene cross-linked with 10% divinylbenzene, (content of amino groups, 4 mmol/g; average pore diameter, 900 Å) was obtained from VE Chemisches

Kombinat Bitterfeld. Glutaryl-L-phenylalanine 4-nitroanilide, benzoyl-DL-arginine 4-nitroanilide, and soluble starch (Zulkowsky) were purchased from Merck, Darmstadt. All other reagents were the purest ones commercially available.

Preparation of the Immobilized Enzymes

TAP-Silica-Chymotrypsin and TAP-Silica-Trypsin. One g of γ-aminopropyl silica (content of amino groups, 0.22 mmol/g) prepared according to (15) was stirred with 1 g of s-trichlorotriazine in 10 ml of acetonitrile containing 0.1 ml of triethylamine at room temperature for 20 min. Then the resin was washed with acetonitrile three times for 5 min and with water six times for 1 min on a glass filter. The sucked activated γ-aminopropyl silica was stirred with 10 ml of enzyme solution (20–100 mg of chymotrypsin or trypsin in 0.1 M phosphate buffer, pH 7.0) at 20°C for 20 min. The product was washed successively with water three times for 5 min, with 30 ml of 0.5 M NH_4OH/1 M NH_4Cl for 30 min (to block the excess reactive groups), and with 0.1 M phosphate buffer/1 M NaCl, pH 7.0 (40 ml in each case) eight times for 30 min. During the last three washing cycles, no protein could be detected in the filtrate. The TAP-silica-enzymes were stored in water at 4°C.

SuAP-Silica-Chymotrypsin and SuAP-Silica-Trypsin One g of γ-succinamidopropyl silica (content of carboxyl groups, 0.21 mmol/g) prepared according to (15) was converted to the *N*-hydroxysuccinimide ester by reaction with 250 mg of *N*-hydroxysuccinimide and 500 mg of *N*-cyclohexyl-*N'*-[β-(*N*-methyl-morpholino)-ethyl]-carbodiimide *p*-toluene sulfonate in 8 ml of dimethylformamide.The activated carrier was stirred with 10 ml of enzyme solution (10–100 mg of chymotrypsin or trypsin in 0.1 M borate buffer, pH 8.4). The SuAP-silica-enzymes were washed and stored as described above.

Silica-Chymotrypsin (Cross-Linked). One g of silica (without preceding functionalization) was stirred with 10 ml of enzyme solution (20–100 mg of chymotrypsin in 0.07 M phosphate buffer, pH 7.0). Nonadsorbed enzyme was separated by washing with water. For cross-linking, the adsorbed enzyme was stirred with 2.5% glutaraldehyde solution in 0.07 M phosphate buffer, pH 7.0, for 2 h. The excess glutaraldehyde was removed by washing with 0.07 M phosphate buffer/1 M NaCl, pH 7.0, for 3 h.

PA-Chymotrypsin. One g of Enzacryl AH was converted into the azide according to Inman and Dintzis (16). The activated gel was stirred with 100 mg of chymotrypsin in 10 ml of 0.1 M borate buffer, pH 8.4, at 3°C for 2 h. During the preparation the pH was kept constant by the addition of 1 M NAOH. Then the gel was treated with 0.5 M NH_4OH/1 M NH_4Cl at 3°C for

2 h and washed with 0.1 M borate buffer/1 M NaCl, pH 8.4. The product was stored in water at 4°C.

QPS-Amylase. The activation of Wofatit Y58 was carried out by the method of Brandt et al. (17) with the following modification: 1 g of Wofatit Y58 was converted to the NH_2 form by treatment with 1 ml of triethylamine in 8 ml of ethanol. After washing with ethanol, the weakly basic anion exchanger was stirred with 400 mg of benzoquinone (purified by sublimation) at room temperature for 1 h and washed with ethanol and ethanol/H_2O (1/1) until the filtrate was free of benzoquinone. The activated anion exchanger was stirred with 10–50 mg of α-amylase in 0.1 M phosphate buffer, pH 7.0, at room temperature for 3 h. The product was washed under stirring with 1 M ethanolamine·HCl, pH 8.0, 0.1 M phosphate buffer/1 M NaCl, pH 7.0, and water. It was stored in water at 4°C.

Protein Determination

The protein content of the silica-enzymes was determined by amino acid analysis (Unichrom, Beckman). The dried samples were destroyed mechanically and hydrolyzed by 5.6 N HCl under air exclusion at 110°C for 24 h. In the calculation the constants of a calibration mixture (Bio Cal) were used. The protein content of PA-chymotrypsin and QPS-amylase was determined from the absorption difference (280 nm) between the initial enzyme solution and the supernatant, including washings after coupling. The absorption coefficients $A_{280}^{1\%} = 19.7$(18) and $A_{280}^{1\%} = 20.0$ (19) were used for α-amylase and chymotrypsin, respectively.

Enzyme Assays

The activities of the enzyme derivatives were determined under stirring (stirrer speed: 1200 rpm) in a water-jacketed vessel containing a glass filter and a valve below the filter bottom. Usually 10–15 mg of the derivative was incubated with 10 ml of substrate solution. The reaction was stopped by sucking off the solution from the derivative. To obtain progress curves, 3–4 samples were assayed with incubation times of 5–30 min.

The substrate solutions were glutaryl-L-phenylalanine 4-nitroanilide in 0.05 M Tris HCl buffer/0.05 M $CaCl_2$, pH 8.0, for the chymotrypsin derivatives, and benzoyl-DL-arginine 4-nitroanilide in the same buffer for the trypsin derivatives. The activities were determined from the increase of the liberated 4-nitroaniline by measuring the absorption (VSU 2, VEB Carl Zeiss, Jena) at 405 nm ($\varepsilon = 10{,}300\ M^{-1}\ cm^{-1}$). The K'_M-values were ascertained from the Lineweaver-Burk plots. The substrate concentration ranged from 0.1 to 1 mM. In the inactivation studies it was 5 mM.

For the assay of QPS-amylase soluble starch (Zulkowsky), 0.025 M acetate buffer, pH 5.5, was used. The activity was ascertained from the increase of reducing groups by measuring the absorption at 530 nm after treatment with 3,5-dinitrosalicylic acid (20). For the determination of the K'_M values, the starch concentration ranged from 5 to 80 mg/ml. In the inactivation studies it was 100 mg/ml.

Thermal Inactivation

The enzyme derivatives (10–15 mg) were preincubated with 2 ml of buffer in closed test tubes at the corresponding temperature. Samples were taken after several periods, quickly cooled, and stored at 4°C until assayed. Storage before the assay (5 min to 20 h) at 4°C or 20°C did not alter the remaining activities. The samples were assayed as described above.

Determination of Enzyme Leakage

Enzyme derivatives, 200–300 mg, in 2 ml of 0.05 M Tris·HCl buffer, pH 8.0 (chymotrypsin and trypsin derivatives) or 0.02 M acetate buffer, pH 5.5 (QPS-amylase), were incubated at 60°C for 40 h. Then the sample was washed with the corresponding buffer containing 1 M NaCl, and the filtrate was filled up to 10 ml. The protein content of the solutions was determined according to Lowry et al (21) by means of calibration curves.

Treatment of Data

The parameters x_i and k_i [Eq. (1)] were determined by a least-square estimation according to a programm (Rechenzentrum, Martin-Luther Universität, Halle) for nonlinear regression.

RESULTS AND DISCUSSION

Characterization of the Enzyme Derivatives

Chymotrypsin and trypsin were bound to γ-aminopropyl silica activated by s-trichlorotriazine (TAP-silica-enzymes) and to succinamidopropyl silica via *N*-hydroxysuccinimide ester (SuAP-silica-enzymes). Another silica-chymotrypsin derivative was obtained by the adsorption of chymotrypsin to nonsubstituted silica and subsequent cross-linking of the enzyme by glutaraldehyde (silica-chymotrypsin, cross-linked). PA-chymotrypsin was synthesized from chymotrypsin and cross-linked polyacrylamide (Enzacryl AH) via azide groups. QPS-amylase was the product of fungal

α-amylase and the polystyrene anion exchanger activated by quinone. The characteristic data of the immobilized enzymes are summarized in Table 1.

The pH optima generally correspond to those of the soluble enzymes. Exceptions are SuAP-silica-chymotrypsin and SuAP-silica-trypsin, for which in comparison to soluble chymotrypsin and trypsin, the pH optima are shifted by 0.3–0.5 pH units into the alkaline range. This pH shifting is caused by the presence of free carboxyl groups on the matrix. The content of free carboxyl groups in the SuAP-silica-enzymes, which was determined by titration, amounts to 0.1 mmol/g enzyme derivative.

In order to obtain comparable values in stability studies, diffusion effects in the enzyme assays had to be excluded (22, 23). The K'_M values shown in Table 1, which are generally higher than those for the soluble enzymes, point to the influence of substrate or product diffusion on the activity of the immobilized enzymes at substrate concentrations below or in the range of the K'_M-values. To eliminate diffusion effects as far as possible, we have carried out the enzyme assays at substrate concentrations which were several times higher than the K'_M values. In addition, the protein content of the carriers was reduced as much as $\frac{1}{4}$ of the protein content given in Table 1. Furthermore, the experiments were repeated with silica-enzymes and polystyrene-amylase, whose particle size was reduced mechanically to <40 μm. All these manipulations did not alter the observed activities A_t/A_0 (Figs. 1–4), so that diffusion effects should not influence the enzyme assays.

TABLE 1. **Characteristic Data of the Prepared Enzyme Derivatives**

Enzyme derivative	Bound protein (mg/g dry carrier)	Activity (μmol/min · g dry derivative)	Preserved activity (%)	K'_M (mM)	pH optimum
TAP-silica-chymotrypsin	38	0.32	20	0.6	8.0
SuAP-silica-chymotrypsin	37	1.36	67	0.91	8.3
Silica-chymotrypsin (cross-linked)	55	0.98	33	2.3	8.0
PA-chymotrypsin	50	2.70	95	0.71	8.0
TAP-silica-trypsin	31	3.40	12	0.95	8.0
SuAP-silica-trypsin	33	11.50	34	0.35	8.3
QPS-amylase	15	205.0	13	35.0[a]	5.0
Chymotrypsin		0.055[b]		0.25	8.0
Trypsin		1.02[b]		0.35	8.0
α-Amylase		105[b]		1.90[a]	5.0

[a] K'_M in g starch/liter.
[b] In μmol · min^{-1} · mg^{-1}.

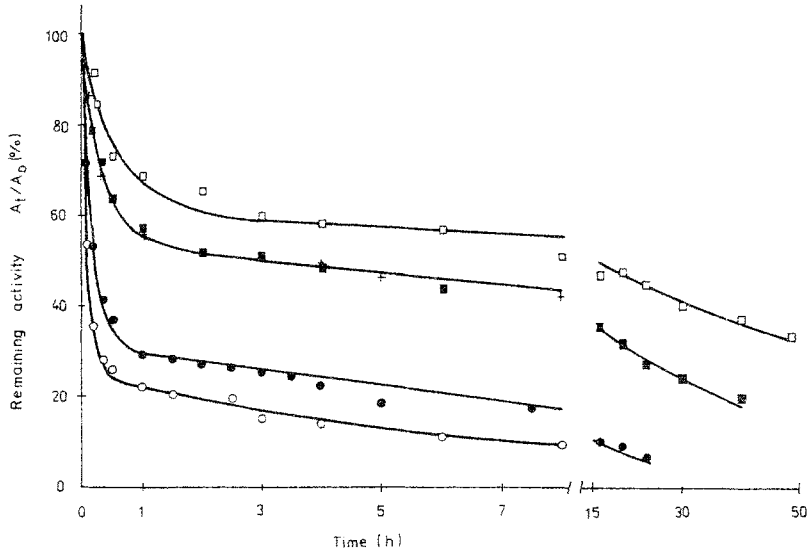

FIG. 1. Inactivation kinetics of the chymotrypsin derivatives at 50°C. The enzyme samples were preincubated in 0.05 M Tris · HCl buffer, pH 8.0, in the presence or absence of 0.05 M $CaCl_2$ for several periods, rapidly cooled, and assayed at 25°C. The remaining activities were related to the corresponding activities without preincubation (for details see the text under Methods). The solid lines represent the inactivation curves calculated according to Eq. (2) for the inactivation of TAP-silica-chymotrypsin in the presence (●) and absence of $CaCl_2$ (○), silica-chymotrypsin (cross-linked) in the presence (□) and absence (■) of $CaCl_2$, and SuAP-silica-chymotrypsin in the absence of $CaCl_2$ (+).

This assumption could also be supported by the evaluation of diffusion effects on the trypsin and chymotrypsin derivatives according to the method of Engasser (24). An external transport coefficient of $h = 3.5 \times 10^{-3}$ cm·s^{-1} and an effective internal diffusional coefficient of $D_{eff} = 3 \times 10^{-6}$ cm^2 · s^{-1} were used, and the effectivity factors were taken from the published nomograms (24). In all cases the product of external and internal effectivity factors was ≥ 0.95.

Another problem in stability studies is the leakage of enzyme from the carrier. This has been observed especially with proteins bound to BrCN-activated carbohydrate carriers (25,26). Experiments concerning enzyme leakage in the derivatives prepared by us (described above under Methods) have shown that never more than 3% of the bound protein was removed from the carrier. The stability of the carrier-enzyme linkage was also confirmed in the preincubation of SuAP-silica-trypsin in the presence of 0.05 M $CaCl_2$. The derivative retained its activity at 50°C for 48 h (Fig. 2).

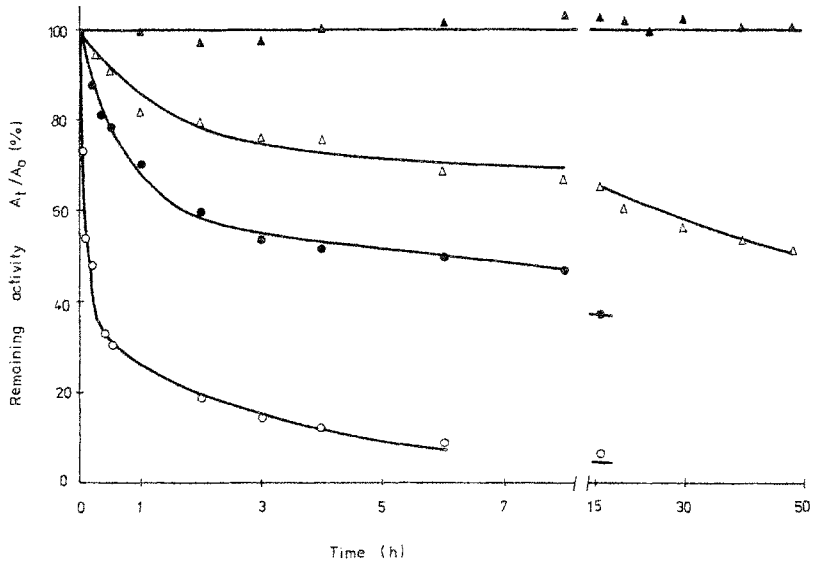

FIG. 2. Inactivation kinetics of the trypsin derivatives at 50°C. The conditions are the same as in Fig. 1. The solid lines represent the inactivation curves calculated according to Eq. (2) for the inactivation of TAP-silica-trypsin in the presence (●) and absence (○) of $CaCl_2$, and SuAP-silica-trypsin in the presence (▲) and absence (△) of $CaCl_2$.

Thermal Inactivation

In Fig. 1 the progress of thermal inactivation is displayed for TAP-silica-chymotrypsin, SuAP-silica-chymotrypsin, and silica-chymotrypsin (cross-linked). It is shown that SuAP-silica-chymotrypsin and silica-chymotrypsin (cross-linked) are more thermostable than TAP-silica-chymotrypsin. After preincubation in 0.05 M Tris · HCl buffer, pH 8.0, at 50°C for 3 h, the remaining activity of SuAP-silica-chymotrypsin and silica-chymotrypsin (cross-linked) amounts to 50%, whereas it decreases to 15% in TAP-silica-chymotrypsin. Surprisingly, the inactivation curves of SuAP-silica-chymotrypsin and silica-chymotrypsin (cross-linked) are nearly identical. Differences in stability like those of the chymotrypsin derivatives, which depend on the binding method, were also observed in SuAP-silica-trypsin and TAP-silica-trypsin (Fig. 2).

From amino acid analysis it was evaluated for TAP-silica-chymotrypsin and TAP-silica-trypsin that, on the average, 4.7–5 lysine residues per protein molecule are covalently bound to the matrix. Unfortunately, such an analysis was not possible for the SuAP-silica derivatives because of the acid-labile amide linkage between protein and carrier. Therefore, the

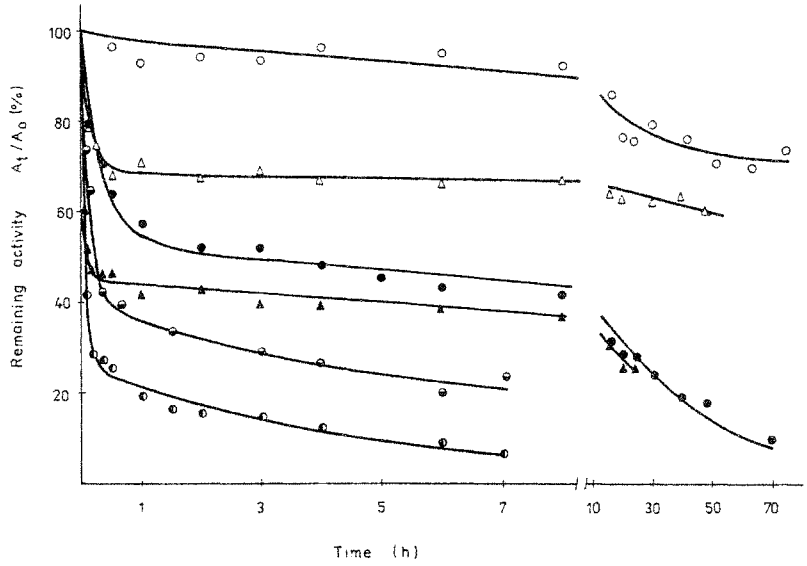

FIG. 3. Inactivation kinetics of the chymotrypsin derivatives at several temperatures (40–60°C). The enzyme samples were preincubated in 0.05 M Tris · HCl buffer, pH 8.0, for several periods, rapidly cooled, and assayed at 25°C. The remaining activities were related to the corresponding activities without preincubation. The solid lines represent the inactivation curves calculated according to Eq. (2) for the inactivation of SuAP-silica-chymotrypsin at 40 (○), 50 (●), 55 (◒), and 60°C (◐); PA-chymotrypsin at 50 (△) and 60°C (▲).

number of covalent linkages and the stability differences observed cannot be correlated. But the higher stability of SuAP-silica-chymotrypsin and SuAP-silica-trypsin compared to the TAP-silica derivatives might be attributed to a higher number of covalent bonds and/or noncovalent interactions between protein and matrix (e.g., between the lysine residues of the protein and the carboxylate groups of the matrix). The stability of silica-chymotrypsin (cross-linked) should result from the inter- and intramolecular cross-links of the protein on the silica surface.

The thermal inactivation of the silica-enzyme derivatives in the presence of Ca^{2+} (Figs. 1 and 2) reflects the well-known conformation-stabilizing effect of calcium ions on these enzymes. In the presence of 0.05 M $CaCl_2$, SuAP-silica-trypsin retains its original activity at 50°C within at least 48 h (Fig. 2).

The inactivation progress in dependence on the temperature is shown for SuAP-silica-chymotrypsin (40–60°C) in Fig. 3 and for QPS-amylase (48–64°C) in Fig. 4. As expected, the stability of the derivatives decreases as the temperature increases. A comparison of the activities remaining after

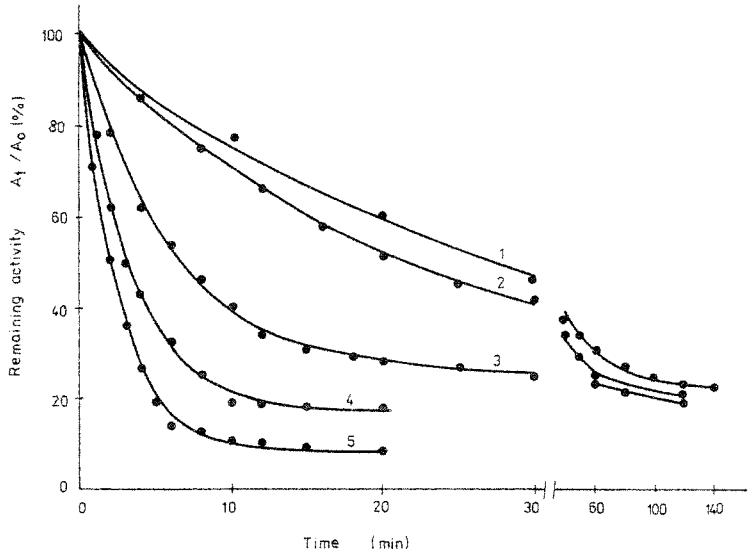

FIG. 4. Inactivation kinetics of QPS-amylase at several temperatures (48–64°C). The enzyme samples were preincubated in 0.025 M acetate buffer, pH 5.5, for several periods, rapidly cooled, and assayed at 37°C. The remaining activities were related to the corresponding activities without preincubation (for details see the text under Methods). The solid lines represent the inactivation curves calculated according to Eq. (2) for the inactivation at 48 (*1*), 52 (*2*), 56 (*3*), 60 (*4*), and 64°C (*5*).

the preincubation of PA-chymotrypsin and SuAP-silica-chymotrypsin at 50 and 60°C shows a higher stability of PA-chymotrypsin after preincubation periods >1 h (Fig. 3). This difference should be based mainly on the different chemical and physical properties of the polyacrylamide and silica matrix.

After preincubation at the different temperatures, the storage of the enzyme derivatives at 4 or 20°C for periods between 2 min and 20 h did not affect the remaining activities. Therefore under the given conditions, the thermal inactivation can be assumed to be irreversible.

Kinetic Model

For a quantitative description of the thermal inactivation behavior of the enzyme derivatives (Figs. 1–4), the parameters x_i and k_i according to Eq. (1) were calculated using a nonlinear least-square program. The calculation started from the assumption of two, three, or four differently stable enzyme populations on the carrier ($i = 2, 3, 4$). The results given in Tables 2 and 3 demonstrate that the inactivation curves are well described by a model

TABLE 2. **Inactivation Constants k_1 and k_2, and Relative Concentration of the Labile Enzyme Fraction x_1 of the Chymotrypsin and Trypsin Derivatives at 50°C**[a]

Enzyme derivative	k_1 (min^{-1})	$k_2 \times 10^4$ (min^{-1})	x_1	SD[b] (%)
TAP-silica-chymotrypsin	0.18	22.1	0.75	3.9
TAP-silica-chymotrypsin ($CaCl_2$)	0.10	12.3	0.69	2.4
SuAP-silica-chymotrypsin	0.055	4.6	0.46	3.5
Silica-chymotrypsin (cross-linked)	0.048	4.2	0.45	3.0
Silica-chymotrypsin (cross-linked) ($CaCl_2$)	0.031	2.2	0.39	3.6
TAP-silica-trypsin	0.20	45.2	0.65	4.0
TAP-silica-trypsin ($CaCl_2$)	0.024	4.9	0.40	3.2
SuAP-silica-trypsin	0.013	1.3	0.26	3.4
Chymotrypsin	0.50			
Chymotrypsin ($CaCl_2$)	7.1×10^{-3}			
Trypsin	0.13			

[a] k_1, k_2, and x_1 were calculated from the remaining activities A_t/A_0 (Figs. 1 and 2) according to Eq. (2).
[b] In this column the standard deviations of the measured remaining activities from the fitted curves are given.

TABLE 3. **Inactivation Constants k_1 and k_2, and Relative Concentration of the Labile Enzyme Fraction x_1 of Chymotrypsin Derivatives and QPS-Amylase at Several Temperatures**[a]

Enzyme derivative	Temperature (°C)	k_1 (min^{-1})	k_2 (min^{-1})	x_1	SD[b] (%)
SuAP-silica chymotrypsin	40	9.0×10^{-4}	9×10^{-12}	0.29	4.1
	50	0.055	4.6×10^{-4}	0.46	3.5
	55	0.080	2.0×10^{-3}	0.62	3.4
	60	0.31	3.4×10^{-3}	0.74	2.5
PA-chymotrypsin	50	0.10	4.5×10^{-5}	0.32	1.5
	60	0.41	4.3×10^{-4}	0.55	2.2
QPS-amylase	48	0.038	7.5×10^{-6}	0.78	1.9
	52	0.047	4.4×10^{-6}	0.79	1.1
	56	0.18	8.0×10^{-6}	0.81	2.0
	60	0.30	1.0×10^{-5}	0.83	1.1
	64	0.40	3.0×10^{-6}	0.92	1.6

[a] k_1, k_2, and x_1 were calculated from the remaining activities A_t/A_0 (Figs. 3 and 4) according to Eq. (2).
[b] In this column the standard deviations of the measured remaining activities from the fitted curves are given.

equation based on only two differently stable populations of bound enzyme molecules [Eq. 2)] with the inactivation constants k_1 and k_2:

$$A_t/A_0 = (x_1)\exp(-k_1 t) + (1-x_1)\exp(-k_2 t) \qquad (2)$$

The standard deviations of the experimental values from the values of the theoretical model amount to only 1–4% (≤experimental error). In Figs. 1–4 the fitted curves are indicated by the solid lines. The use of the model equation [Eq. (1)] with $i = 3$ or 4 does not improve the mathematical approach. In some cases the standard deviations become larger; in others identical inactivation constants were obtained, or the fraction of a third or fourth postulated enzyme population was less than 8%. Therefore, the model with two differently stable enzyme populations proved to be sufficient to describe the thermal inactivation behavior of all the different types of immobilized enzyme derivatives.

The inactivation of the corresponding soluble enzymes (not shown in the figures) can be described by first-order kinetics (Table 2). The thermal inactivation of soluble chymotrypsin and trypsin at 50°C, however, obeys a first-order reaction only up to 20% and 12% remaining activity. The deviation at lower activities may be caused by autolysis or protein association. In contrast, the first-order inactivation kinetics of amylase are observed in the temperature range 44–60°C up to a remaining activity of 5%.

A biphasic course in the thermal inactivation of immobilized enzymes has also been discussed by other authors (6,7). It can be supposed that the conformational stability of a part of the enzyme molecules is increased by covalent or noncovalent multipoint attachment, whereas the other part is in a conformation similar to that of the soluble enzyme. In the inactivation of QPS-amylase, there is a good agreement of the inactivation constant k_1 and the activation energy of inactivation ($E_A = 178$ kJ/mol) with the corresponding parameters of the soluble amylase ($E_A = 192$ kJ/mol) (27) (Fig. 5). Therefore, the conformation of most active enzyme molecules in QPS-amylase ($x_1 \approx 0.8$) does not seem to be affected by immobilization. Likewise, Koch-Schmidt and Mosbach (3) have observed a nearly identical behavior of ribonuclease bound to Sepharose by only one linkage per protein molecule and the soluble enzyme in thermochemical studies. But in the chymotrypsin and trypsin derivatives there is no correlation between k_1 and the inactivation constants of the soluble enzymes (Table 2). Nevertheless, the thermal inactivation of these derivatives is well described by Eq. (2).

Further information on the thermal inactivation of the immobilized enzymes was obtained from the inactivation curves in dependence on temperature (Figs. 3 and 4). The values in Table 3 show that not only the inactivation constants but also the relative concentrations x_i are dependent

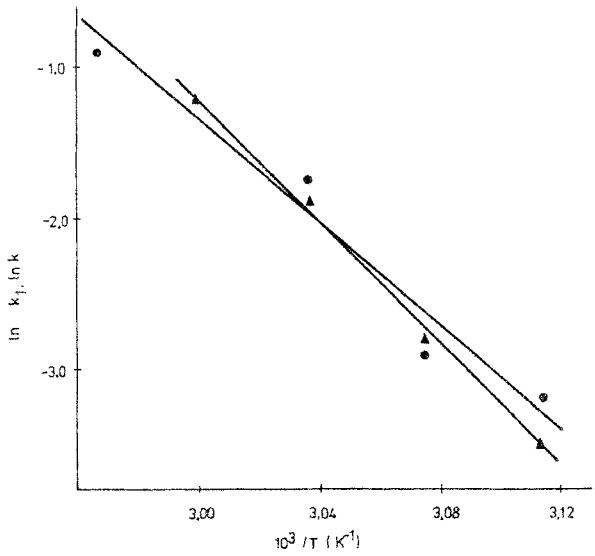

FIG. 5. Arrhenius plot of the inactivation constants k_1 of QPS-amylase (●) and the inactivation constants k of soluble amylase (▲). The k_1 values were taken from Table 3. The inactivation constants for soluble amylase were described previously (27).

on temperature. As the temperature increases, the labile enzyme fraction x_1 increases, and correspondingly the stable fraction decreases.

It is interesting that the distribution between stable and labile fractions, which was studied for SuAP-silica-chymotrypsin and QPS-amylase in dependence on temperature, can be described by the Boltzmann distribution law (Fig. 6). A deviation was observed only with QPS-amylase at 64°C. The energies for the transition from the stable to the labile enzyme form show that these transitions depend more on temperature in SuAP-silica-chymotrypsin ($E = 42$ kJ/mol) than in QPS-amylase ($E \approx 4$ kJ/mol). To interpret this effect we suppose that in SuAP-silica-chymotrypsin especially, noncovalent protein-matrix interactions are responsible for stabilization. As the temperature increases, the stabilizing forces are reduced, or unfavorable interactions decreasing the enzyme stability are formed. In QPS-amylase only small changes of the relative concentrations are observed (Table 3; Fig. 6) so that noncovalent interactions obviously play an unessential role. The agreement of the k_1 values with the values of the soluble enzyme (Fig. 5) and the small change of the k_2 values at different temperatures confirm this explanation (Table 3).

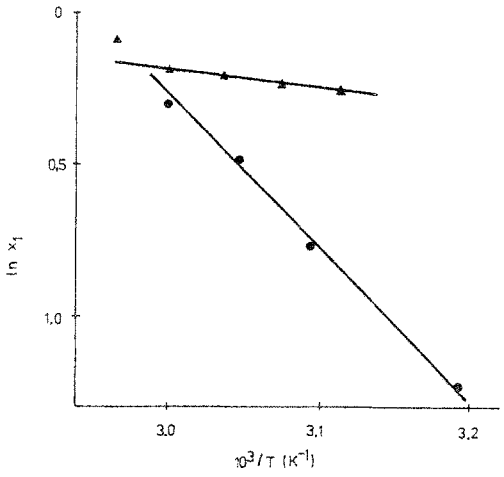

FIG. 6. Plot of $\ln x_1$ against $1/T$ for SuAP-silica-chymotrypsin (●) and QPS-amylase (▲). The relative concentrations x_1 of the labile enzyme fractions were taken from Table 3.

CONCLUSIONS

The biphasic inactivation kinetics usually observed with immobilized enzymes has been described by assuming that the immobilization results in differently stable enzyme fractions, each of which, like soluble enzymes, is inactivated by a first-order reaction. The results have shown that the assumption of only two enzyme fractions with the inactivation constants k_1 and k_2 provides a sufficient description of the inactivation curves. Two enzyme fractions (x_1, x_2), differing remarkably in stability are, therefore, determining for the inactivation process. Apparently, in the immobilization, stabilized and nonstabilized enzyme molecules occur, the relation of which (x_1/x_2) alters with temperature. For interpretation the further investigation of the alteration of this relation by temperature and other factors should be interesting. It must be noted, however, that x_1/x_2 represents the relation of true enzyme concentrations only if the catalytic reaction constants of the different enzyme fractions are identical.

REFERENCES

1. GOLDMAN, R., GOLDSTEIN, L., and KATCHALSKI, E. (1971) *In* Biochemical Aspects of Reactions on Solid Supports, STARK, G. A. (ed.), Academic, New York, pp. 1–78.
2. GABEL, D. (1973) Eur. J. Biochem. 33 : 348–356.

3. KOCH-SCHMIDT, A.-C., and MOSBACH, K. (1977) Biochemistry 16 : 2105–2109.
4. MARTINEK, K., KLIBANOV, A. M., GOLDMACHER, V. S., and BEREZIN, I. V. (1977) Biochim. Biophys. Acta 485 : 1–12.
5. KOCH-SCHMIDT, A.-C., and MOSBACH, K. (1977) Biochemistry 16 : 2101–2105.
6. ZABORSKY, O. R. (1973) *In* Immobilized Enzymes in Food and Microbial Processes, OLSON, A. C., and CONNEY, C. L. (eds.), Plenum, New York, pp. 187–203.
7. UY, R., LIU, V. S. H., and ROYER, G. P. (1976) J. Solid-Phase Biochem. 1 : 51–65.
8. CARDOSO, J. P., and EMERY, A. N. (1978) Biotechnol. Bioeng. 20 : 1471–1477.
9. GABEL, D., and AXEN, R. (1976) Methods Enzymol. 44 : 383–393.
10. FISCHER, J., LANGE, L., KOCH, S., and JAKUBKE, H.-D. (1978) Eur. J. Biochem. 88 : 445–452.
11. FISCHER, J., LANGE, L., and JAKUBKE, H.-D. (1978) Eur. J. Biochem. 88 : 453–457.
12. LAIDLER, K. J., and BUNTING, P. S. (1973) The Chemical Kinetics of Enzyme Action, Clarendon Press, Oxford, p. 416.
13. BENDER, M. L., BEGUE-CANTON, M. L., BLAKELEY, R. L., BRUBACHER, L. J., FEDER, J., GUNTER, C. R., KEZDY, F. J., KILLHEFFER, J. V., MARSHALL, T. H. MILLER, C. G., ROESKE, R. W., and STOOPS, J. K. (1966) J. Am. Chem. Soc. 88 : 5890–5913.
14. CHASE, JR., T., and SHAW, E. (1967) Biochem. Biophys. Res. Commun. 29 : 508–514.
15. LYNN, M. (1975) *In* Immobilized Enzymes, Antigens, Antibodies, and Peptides, WEETALL, H. H. (ed.), Marcel Dekker, New York, pp. 1–48.
16. INMAN, J. K., and DINTZIS, H. M. (1969) Biochemistry 8 : 4074–4082.
17. BRANDT, J., ANDERSSON, L.-O., and PORATH, J. (1975) Biochim. Biophys. Acta 386 : 196–202.
18. FISCHER, E. H., and STEIN, E. A. (1960) *In* The Enzymes, BOYER, P. D., LARDY, H., and MYRBÄCK, K. (eds.), Academic, New York, p. 313.
19. DIXON, G. H., and NEURATH, H. J. (1957) J. Biol. Chem. 225 : 1049–1059.
20. BERNFELD, P. (1955) Methods Enzymol. 1 : 149–158.
21. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951) J. Biol. Chem. 193 : 265–280.
22. OLLIS, D. F. (1972) Biotechnol. Bioeng. 14 : 871–884.
23. ENGASSER, J.-M., and COULET, P. R. (1977) Biochem. Biophys. Acta 485 : 29–36.
24. ENGASSER, J.-M. (1978) Biochim. Biophys. Acta 526 : 301–310.
25. LASCH, J., and KOELSCH, R. (1978) Eur. J. Biochem. 82 : 181–186.
26. MIRON, T., CARTER, W. G., and WILCHEK, M. (1976) J. Solid-Phase Biochem. 1 : 225–236.
27. FISCHER, J., ULBRICH, R., and SCHELLENBERGER, A. (1978) Acta Biol. Med. Ger. 37 : 1413–1424.

APPENDIX: GRAPHIC DETERMINATION OF THE INACTIVATION CONSTANTS OF IMMOBILIZED ENZYMES[2]

In the above discussion it has been shown that the kinetics of the thermal inactivation of immobilized enzymes can be described by the equation

$$A_t/A_0 = x_1\, e^{-k_1 t} + (1 - x_1)\, e^{-k_2 t} \qquad \text{(A1)}$$

[2]This section is by Renate Ulbrich, Volker Pluschke, and Alfred Schellenberger.

A_t is the enzyme activity after the heating time t, A_0 is the activity before heating, k_1 and k_2 are the rate constants of the inactivation of the labile and stable enzyme fractions, and x_1 is the relative concentration of the labile enzyme fraction. Under the condition that the inactivation constants differ by at least one order of magnitude, the parameters k_1, k_2, and x_1 can be approximatively determined by a simple graphic method. In an analogous way, Ling and Lund[3] have analyzed the thermal inactivation of peroxidase isoenzymes. In the present contribution an error estimation has been performed to indicate the conditions under which the graphic approximation is sufficient.

Since the activity of the labile enzyme fraction $x_1 e^{-k_1 t}$ approaches zero more rapidly than that of the stable fraction, for long heating times the activity decrease can be approximated by a straight line:

$$\log(A_t/A_0) \approx \log(1-x_1) - k_2 t/2.303 \qquad \text{(A2)}$$

In the plot of $\log(A_t/A_0)$ against the heating time t the slope and vertical intercept give k_2 and $(1-x_1)$ (Fig. A1). k_1 can be determined by a second graph (Fig. A2). Since the inactivation of the stable fraction can be neglected during the initial phase of inactivation ($k_2 \ll k_1$; $e^{-k_2 t} \approx 1$), for short times the plot of $\log[A_t/A_0-(1-x_1)]$ against the heating time can be approximated by a straight line

$$\log[A_t/A_0-(1-x_1)] \approx \log x_1 - k_1 t/2.303 \qquad \text{(A3)},$$

where the slope provides k_1.

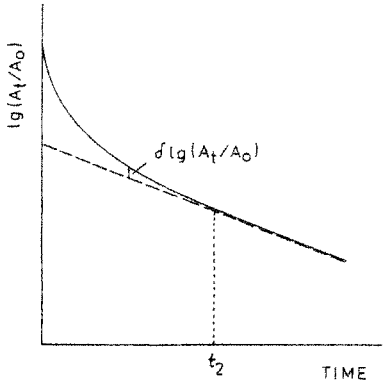

FIG. A1. Determination of k_2 and $(1-x_1)$. The solid curve is experimental; the dashed curve is the approximate straight line from Eq. (A2).

[3]LING A. C., and LUND, D. B. (1978) J. Food Sci. 43: 1307–1310.

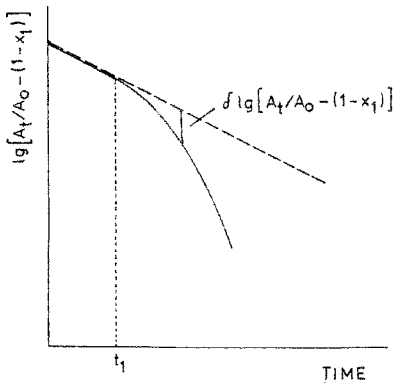

FIG. A2. Determination of k_1. The solid curve is experimental; the dashed curve is the approximate straight line, from Eq. (A3).

The accuracy of the graphic method depends on the number of measurements during the periods in which the approximation of the curves by straight lines is possible. By means of the following error estimations these periods can be determined. Thus Eq. (A1) is transformed into Eq. (A4):

$$\log(A_t/A_0) = \log\left[x_1\, e^{-k_1 t} + (1-x_1)\, e^{-k_2 t}\right]$$

$$= \log\left\{(1-x_1)\, e^{-k_2 t}\left[1+\frac{x_1}{1-x_1}\,e^{-(k_1-k_2)t}\right]\right\}$$

$$= \log(1-x_1) - k_2 t/2.303 + \log\left[1+\frac{x_1}{1-x_1}\,e^{-(k_1-k_2)t}\right] \qquad \text{(A4)}$$

$$\underbrace{\qquad\qquad}_{\text{(A4')}} \qquad\qquad \underbrace{\qquad\qquad}_{\text{(A4'')}}$$

where the expression (A4′) corresponds to Eq. (A2) (Fig. A1) and the expression (A4″) represents the deviation $\delta \log(A_t/A_0)$ of the experimental curve $\log(A_t/A_0)$ from the approximate straight line [Eq. (A2)]. If we require $\delta \log(A_t/A_0) < 0.01$, which is in the range of measuring and drawing errors, the time t_2 (Fig. A1), from which the inactivation curve is replaceable by a straight line, can be estimated from (A4″). With the estimation

$$\log(1+x) < \frac{x}{2.303}$$

it follows that

$$\delta \log(A_t/A_0) = \log\left[1+\frac{x_1}{1-x_1}\,e^{-(k_1-k_2)t}\right] < \frac{x_1}{2.303\,(1-x_1)}\,e^{-(k_1-k_2)t} < 0.01 \qquad \text{(A5)}$$

By use of the simplifications

$$k_1 - k_2 \approx k_1, \quad \text{since } k_2 \ll k_1 \tag{A6}$$

and

$$\frac{x_1}{1-x_1} < 9, \quad \text{if } 0.1 \leq x_1 \leq 0.9 \tag{A7}$$

Eq. (A5) becomes

$$\delta \log(A_t/A_0) < \frac{9}{2.303} e^{-k_1 t} < 0.01 \tag{A8}$$

so that

$$k_1 t > 6 \tag{A9}$$

Therefore, the inactivation curve can be described by a straight line from $t_2 = 6/k_1$ [Eq. (A8)]. For a reliable construction of the straight line, the experiments should be continued up to $t > 15/k_1$ [Eq. (A9)]. In order to determine this period before the beginning of the experiments, when k_1 is not yet known, k_1 in Eqs. (A8) and (A9) can be substituted for by the inactivation constant of the soluble enzyme k_s, because there are generally only small differences between k_1 and k_s.

An analogous error estimation provides the time t_1 (Fig. A2), up to which the curve $\log[A_t/A_0 - (1-x_1)]$ can be replaced by the approximate straight line of Eq. (A3). If the deviation of the experimental curve from the straight line is again < 0.01,

$$\delta \log[A_t/A_0 - (1-x_1)] < \frac{1-x_1}{2.303 x_1} k_2 t e^{k_1 t} < 0.01 \tag{A10}$$

is obtained. Since $(1-x_1)$ and k_2 are known from the first graph, and k_1 can be replaced by k_s for estimation, the relation (A10) provides t_1. Therefore, there should be at least five measurements in the period from $t = 0$ to $t = t_1$. In contrast to t_2, t_1 cannot be predicted before the experiment, but only after the determination of $(1-x_1)$ and k_2 it has to be proved whether the measurements are sufficient or have to be completed.