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A PHYSICO-CHEMICAL ANALYSIS OF SOLUBLE AND IMMOBILIZED ENZYME STABILIZATION

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The literature and our experimental data on the effect of chemical modification and immobilization on the thermostability of enzymes are analyzed. The effect of various factors causing changes in the stability of enzymes after their modification or immobilization is demonstrated. It is shown that changes in the temperature dependence of the inactivation rate constant are associated with the change in the effective values of thermodynamic activation parameters for the inactivation processes. An increase in the activation energy of thermoinactivation, E_a, leads to the stabilization of a modified or immobilized enzyme at temperatures below the iso-kinetic temperature ("low-temperature" stabilization) and a decrease in E_a entails a "high-temperature" stabilization of enzymes. It is shown that with immobilized enzymes the high-temperature stabilization is invariably observed.

INTRODUCTION

Immobilization of enzymes and enzyme systems, as well as their stabilization against thermoinactivation, is of great practical and theoretical importance. Elucidation of the basis of stabilization will, on the one hand, facilitate the problem of application of bioorganic catalysts in chemical technology and, on the other hand, help to comprehend the structure–function relationship in biological macromolecules.

In the recent years, along with extensive studies in enzyme engineering, considerable success has been achieved in the development of the methods of stabilization for soluble and immobilized enzymes. A distinguishing feature of these studies is the tendency not only to register the effect of stabilization but also to elucidate the physico-chemical principles of enzyme stabilization.

Analysis of the papers dealing with thermostability of soluble and immobilized enzymes is hardly possible without defining the very notion of "stabilization" of enzymes. In the majority of papers, the rate constant of monomolecular inactivation, k_{in}, of the enzyme at one temperature is assumed to be a measure of thermostability. Increase or decrease in k_{in}

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testifies to destabilization or stabilization of an enzyme. For each enzyme, the rate constant of thermoinactivation, k_{in}, depends on temperature, and any action produced on the enzymes, for example, chemical modification, change in the composition of the solution, entrapment into a polymeric gel, or immobilization, alters the character of the temperature dependence of k_{in}. Besides, for the majority of enzymes, the dependence of log k_{in} on the inverse temperature is only valid over a narrow temperature range, because of complexity of the conformational conversions occurring in protein when the temperature is changed (1). That is why the conclusion about stabilization, which is inferred from a comparison of the inactivation rate constants at one temperature for a native and immobilized enzyme, can be true of this temperature only (or over a certain narrow temperature range), and the quantitative effect of stabilization changes with temperature. As to the activated complex theory, its applicability to biological macromolecules (2) is often rightly questioned. One cannot help admitting, however, that, with the lack of the workable kinetic theory of enzymatic reactions, one has to resort to Arrhenius plots for a phenomenological description of temperature dependences of inactivation rate constants. In terms of this theory, k_{in} is determined by free activation energy of thermoinactivation, $\Delta G^{\neq}$ (1).

$$\ln k_{in} = \ln k_B \cdot T/h - (\Delta G^{\neq}/RT)$$

The higher the value of $\Delta G^{\neq}$, the more stable the enzyme is (lower k_{in}). From the temperature dependence of log k_{in} on the inverse temperature, the activation energy of the thermoinactivation is found:

$$E_a = 2.303 \cdot \mathrm{R}\,(\log k_{in,1} - \log k_{in,2})/[(1/T_2) - (1/T_1)]$$

and also values

$$\Delta H^{\neq} = E_a - RT, \qquad \text{and} \qquad \Delta S^{\neq} = (\Delta H^{\neq} - \Delta G^{\neq})/T$$

In a general case when temperature dependence changes, one can observe:

(1) An increase in E_a, and hence increase in $\Delta H^{\neq}$ occurs. Thereby, depending on the changes in $\Delta S^{\neq}$, dependences of log k_{in} on $1/T$ for a native and modified enzyme intersect outside or within the temperature range studied. The modified enzyme will be more stable than the native one at the temperatures lower than the isokinetic temperature (T_{isokin}). The effect of stabilization will increase with a decrease in temperature. In this case the so-called low-temperature stabilization will be observed.

(2) A decrease in E_a and consequently, in $\Delta H^{\neq}$, leads to a high-temperature stabilization. At the temperatures higher than T_{isokin}, with an increase in the temperature, the effect of stabilization (i.e., the ratio of the

inactivation constants of the native and modified enzymes) will increase.

In the case when T_{isokin} lies outside of the studied temperature range, stabilization (or destabilization) of the enzyme is observed over the whole range of the experimental temperatures used.

Examples given below show that both the low-temperature and high-temperature stabilization (3,4,5) can be encountered.

The change in enzyme stability after their covalent (or noncovalent) binding with a support (soluble or insoluble) depends on several factors, which can be grouped in the following way: (1) chemical modification of the functional side groups of protein used for the covalent binding of the enzyme to the support; (2) polyfunctional interaction of the enzyme globule with the support, which involves the formation of additional covalent (or noncovalent) linkages and leads to rigidification of the protein structure. This type of interaction has been given the name of "multipoint interaction" (6); (3) restriction of the conformational mobility of the polypeptide chains of the enzyme molecule at the expense of intramolecular cross-linking (7,8) or by entrapping the macromolecule into "tight" pores of polymeric gels (9); (4) change in the microenvironment of the enzyme that is attached to a soluble or an insoluble support (micro pH, dielectric permeability, the difference between the hydrophilic–hydrophobic properties of the groups, that come into contact with the enzyme and those of water, and so forth (10).

In most cases, these factors are at work simultaneously, and moreover, can aim at different targets. Correct estimation of the relative contribution of the various factors to the effect of stabilization (or destabilization) makes possible purposeful choice of different ways and methods of stabilization and immobilization for given enzymes.

Change in the enzyme thermostability on chemical modification by low-molecular weight monofunctional agents was reported some time ago (11). Increase in the thermostability was reported for dye-modified β-galactosidase (12), for pancreatic inhibitor modified by simm-chlortriazine (13), for peroxidase modified by anhydrides of mono- and dicarboxylic acids (13,14).

The latter example should be analyzed in some detail. In Figure 1 there is $\log k_{in}$ of native peroxidase and of peroxidase in which four ε-amino groups are modified by anhydrides of mono- and dibasic acids. In all cases, the modified enzyme is several orders more stable than the native one (50°, pH 7.0). At the same time the nature of radical R does not greatly affect the thermostability of the modified enzyme.

However, the data obtained at one temperature are not sufficient for the effect of chemical modification on the thermostability of a given enzyme to be fully understood. Figure 2 shows the dependences of $\log k_{in}$ on the inverse temperature for native peroxidase (Fig. 2, curve 1) and that modified

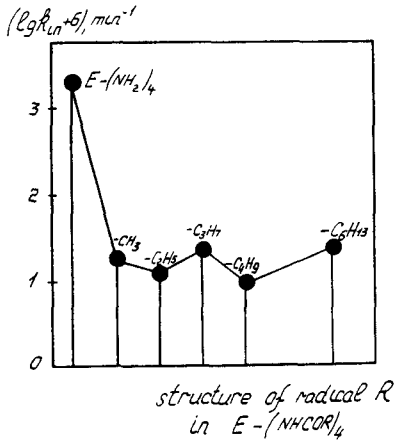

FIG. 1. Thermostability of peroxidase, native and modified by anhydrides of mono- and dibasic acids. The effect of the structure of a radical, R, introduced on modification, on the inactivation rate constant of the enzyme at 50°C, pH 7.0 [14].

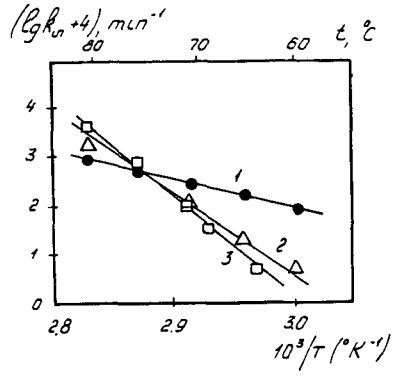

FIG. 2. Temperature dependence of the inactivation rate constant of peroxidase: native (1); modified by propionic anhydride (2); and by succinic anhydride (3), at 60 to 80°C.

by propionic and succinic anhydrides (Fig. 2, curves 2 and 3). There is an intersection of the Arrhenius plots at a point corresponding to 74°C (T_{isokin}), under which the modified enzyme is more stable and above which the native enzyme is more stable. This case is an example of the "low-temperature" stabilization. For modified peroxidase, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ of the thermoinactivation sharply increase (Fig. 3) and the change of both parameters is almost parallel.

Consequently, acylation of four amino groups of peroxidase increases both $\Delta H^{\neq}$ and $\Delta S^{\neq}$ of the irreversible thermoinactivation. A two-fold increase in $\Delta H^{\neq}$ produces a stabilization effect, whereas an increase in $\Delta S^{\neq}$ produces a destabilization effect. The observed stabilization effect should be ascribed to the enthalpy member, that is, to the increase in $\Delta H^{\neq}$.

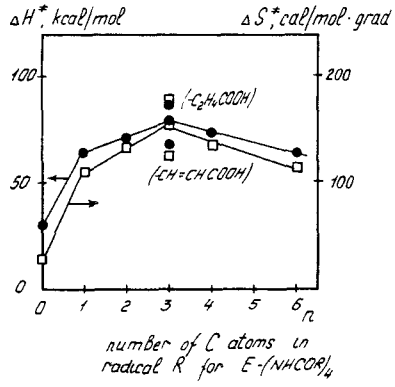

FIG. 3. The effect of the structure of a radical introduced on acylation of peroxidase on the thermodynamic activation parameters of irreversible thermoinactivation (14). In parentheses, the structure of radical R.

What is the effect of the nature of radical R, introduced into the enzyme on its being acylated?

$$E(NH_2)_4 + 4(RCO)_2O \rightarrow E(NHCOR)_4 + 4RCOOH$$

When $R = C_3H_7$ is used instead of $R = CH_3$, and $R = C_2H_5$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ increase, but for $R = C_4H_9$ and $R = C_6H_{13}$ they decrease. The negative charge on R considerably increases the values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ (compare modification by butyric and succinic anhydrides). With maleic anhydride, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ are much lower than for succinic anhydride. This seems to be due to the unfavorable interaction of the more rigid maleic acid residue with the enzyme globule.

Arylation of amino groups by trinitrobenzenesulfo acid (TNBS) produces a much less significant effect on the thermostability of peroxidase. As shown in Figure 4, for the enzyme with three arylated groups, there is a certain stabilization at temperatures beneath 75°C. With all the six ε-amino groups being arylated, the thermostability of the enzyme even decreases somewhat. For peroxidase with three TNBS-modified groups, an increase in

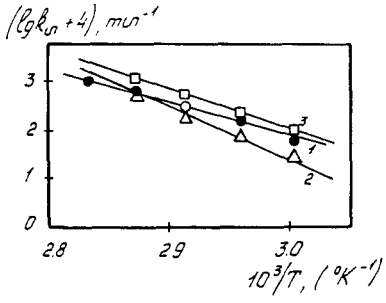

FIG. 4. Temperature dependences of the thermoinactivation rate constant of peroxidase: native (1) and modified by trinitrobenzenesulfo acid (2–three groups, 3–six groups were modified) (14).

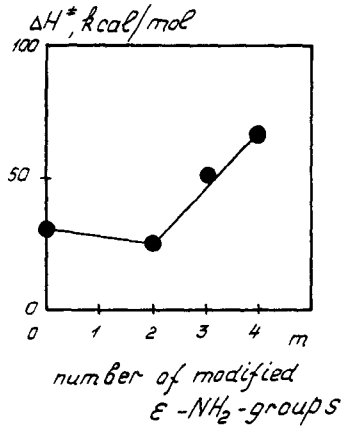

FIG. 5. $\Delta H^{\neq}$ of irreversible thermoinactivation of peroxidase modified by enanthic anhydride as a function of the number of the modified ε-amino groups (m).

$\Delta H^{\neq}$ and $\Delta S^{\neq}$ is observed, although much less than for the anhydride-modified enzyme. For the enzyme containing six TNBS-modified groups, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ hardly differ from the corresponding parameters of the native enzyme. Hence, the stabilization effect depends not only on the nature of a radical introduced into an enzyme, but on the number of modified functional groups; this is exemplified in Figure 5 with peroxidase modified by enanthic anhydride. As the number of modified ε-amino groups increases, $\Delta H^{\neq}$ of the irreversible thermoinactivation increases as well proportionally to the number of the modified functional groups.

Change in the thermostability of the modified enzyme as compared to that of the native one is first of all due to changes in its conformation. This suggestion is substantiated by the CD spectra of native and modified peroxidase (Fig. 6). For modified peroxidase, the optical activity of the haem

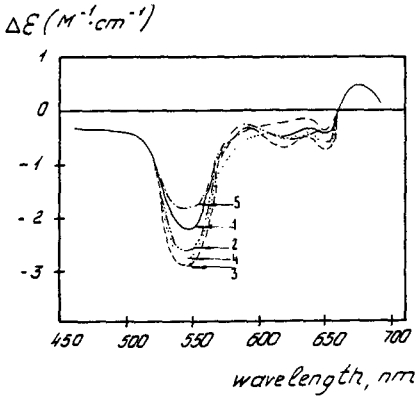

FIG. 6. CD spectra of peroxidase, native and modified, in the 470 to 700 nm range (15). Symbols: native peroxidase, 1, modified by butyric anhydride, 2, by succinic anhydride, 3, by trinitrobenzenesulfo acid, 4, 5 (three and six groups are modified, respectively). Conditions: 0.05 M NaCl, 22°C.

bound to protein (λ_{max} = 540, 620, and 652 nm) markedly changes (15). The character of these changes is similar to those in thermostability of the enzyme after modification. A decrease in the thermostability of peroxidase with six TNBS-modified ε-amino groups is accompanied by a decrease in $\Delta\varepsilon$ in the CD-spectra of the haem. On the contrary, an increase in $\Delta\varepsilon$ is observed after modification of peroxidase by anhydrides, which leads to the stabilization of this enzyme. As the length of R increases to a certain extent, favorable conformational changes occur in the protein, which increase its stability. On further elongation of R, exposure of the hydrophobic residue to the solution becomes energetically unfavorable and it is "drawn" inside the globule, which "loosens" the latter. Hence, moderate "hydrophobization" of the protein globule can lead to its stabilization. This conclusion was arrived at in the works on the solubilization of hydrocarbons by proteins (16).

Chemical modification of the functional groups in proteins by bifunctional agents leads to the formation of intramolecular cross-linkages which should entail the rigidification of the protein globule, a decrease in $\Delta S^{\neq}$, and, consequently, an increase in the free activation energy of thermoinactivation. Bifunctional agents used for enzyme modification are dialdehydes, diimidoesters, diisocyanates (17). But alongside the formation of monomeric molecules of the enzyme with intramolecular linkages, formation of oligomeric proteins is not impossible, which is often overlooked. An interesting approach to obtaining intramolecular linkages in the enzyme was suggested in (8). The authors used diamines to form intramolecular amide bonds in enzymes whose carboxylic groups were preactivated by soluble carbodiimides. The effect of stabilization depends on n, that is, the number of methylene groups in the molecule of diamine. For chymotrypsin, maximal stabilization is achieved at $n = 4$. The authors believe that it is the length of the tetramethylene-diamine molecule that best fits the distance between the carboxylic groups of the protein molecule and gives the optimal number of the intramolecular cross-linkages. The effect of stabilization increases, if chymotrypsin is pretreated by succinic anhydride. Thereby part of the ε-amino groups convert into carboxylic groups, and the occupancy of the surface of the protein globule by carboxylic groups increases as compared to the native enzyme. In this case, the maximal stabilization is observed when a shorter bifunctional agent, ethylenediamine, is used. Stabilization of enzymes by intramolecular cross-linking can be explained not only in terms of "mechanical fixation" of the native conformation of the protein in solution. Perhaps there are also conformational changes of protein modified by reagents of a different chemical nature.

Stabilization of water soluble enzymes is often achieved by covalent binding with water soluble supports. In this case different factors could be at

work, which can determine the enzyme thermostability, for example, chemical modification of protein, the polyfunctional multipoint interaction of the enzyme with a polymer, a change in the environment, mechanical rigidification of the enzyme molecule due to the fixation of that of a polymer on or around the protein globule.

In the literature, the stabilization effect is mostly attributed to the second factor. However, some recently published data show that other factors can predominate. For example, in (13) it is shown that the stabilization of the trypsin pancreatic inhibitor covalently bound to dextran via chlortriazine occurs owing only to the modification of tyrosine groups of the protein by the binding agent. Subsequent covalent binding of dextran does not change the thermostability of the protein.

Soluble polymers of the most diverse nature are used for the binding with enzymes, for example, dextrans (18,19), modified Sephadexes (20), carboxymethylcellulose (4), vinyl series copolymers (21), proteins (albumin) (3), and so forth. Analysis of the thermodynamic activation parameters for the inactivation of peroxidase covalently bound to albumin (3), and for chymotrypsin bound to soluble CM-cellulose [4] shows that in this case enzyme stabilization can be associated both with an increase in the enthalpy of activation, $\Delta H^{\neq}$, and, mainly, with a decrease in the entropy of activation, $\Delta S^{\neq}$. For peroxidase-albumin oligomers, $\Delta H^{\neq}$ of inactivation increases almost two-fold, which compensate for the increase in $\Delta S^{\neq}$ of the process. The total effect of stabilization reaches several orders of magnitude. For the stable fraction of CMC-chymotrypsin (Fig. 7b), a strong decrease in $\Delta H^{\neq}$

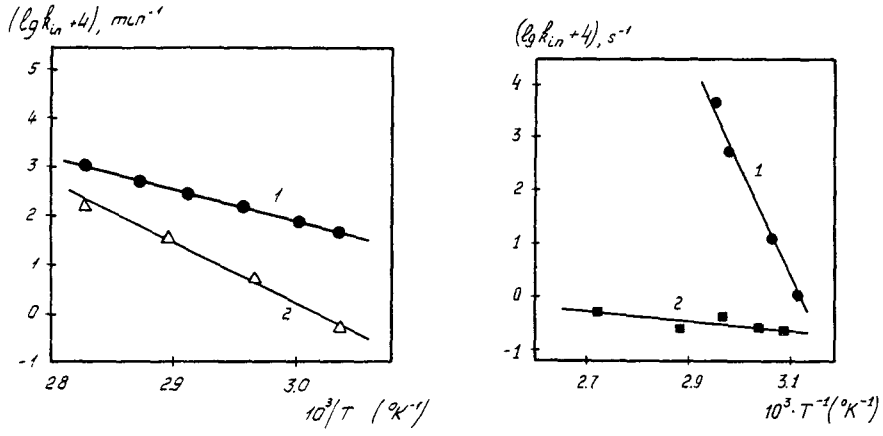

FIG. 7. Temperature dependences of the thermoinactivation rate constant of native and modified enzymes. (a, left) Peroxidase (3): native, 1, covalently attached to albumin, 2; (b, right) Chymotrypsin (4): native, 1, covalently bound to soluble CM-cellulose, 2.

(from 57 kcal/mol for the native enzyme to 1.5 kcal/mol for CMC-chymotrypsin) is compensated for by a high entropy contribution to the stabilization. $\Delta(\Delta S^{\neq})$ is as high as 200 eu (4). The stabilization effect reaches many orders of magnitude.

Analysis of the problem of the stabilization of insoluble immobilized enzymes, reveals similar regularities in the change of the thermodynamic activation parameters. In enzymes covalently bound to an insoluble support or entrapped into "tight" pores of a polymer gel, the conformational mobility is greatly restricted. After immobilization, $\Delta H^{\neq}$ usually decreases (4,5,22); therefore $\Delta S^{\neq}$ sharply decreases. Hence, stabilization of the immobilized enzymes is commonly due to the effect of the entropy member, $\Delta S^{\neq}$. It is mostly with immobilized enzymes that the "high-temperature" stabilization manifests itself, that is, at temperatures beyond T_{isokin}. Figure 8 shows the temperature dependences of log k_{in} of the inactivation rate constant for the native chymotripsin in solution (Fig. 8, curve 1) and chymotripsin entrapped into polyacrylamide gel (PAAG) of different concentrations (5). As the concentration of PAAG increases from 48 to 100%, the effect of stabilization increases millions of times due to an enormous decrease in $\Delta S^{\neq}$. This decline is a likely result of the decrease in the conformational mobility of the protein globule. Entrapment into high concentration gels (gels of higher than 60% concentration were obtained by dehydration of 55% gel) resembles lyophilization (cf. the temperature dependences of k_{in} of lyophilized chymotrypsin, Fig. 8, curve 2). However, 100% PAAG gives a still stronger effect since the conformational mobility of macromolecules in the pores of the gel is even lower than that in a crystal.

Factors, such as the chemical nature of a support surface and a cross-linking agent and the nature and degree of modification of the protein functional group participating in the binding with the support, produce a significant effect on the thermostability of the immobilized enzyme. In the case when stabilization is achieved by interaction with a support, the elongation of the "spacer" through which the enzyme is attached to the

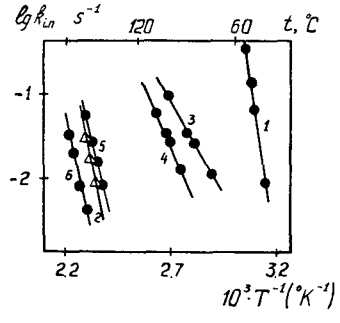

FIG. 8. Temperature dependences of the thermoinactivation rate constant of chymotrypsin (5). Symbols: native enzyme in solution, 1; native lyophilized, 2; entrapped into polyacrylamide gel, gel concentrations: 48.5%, 3; 60%, 4; 80%, 5; 100%, 6.

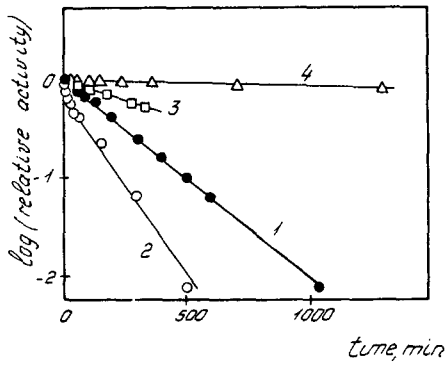

FIG. 9. Semilogarithmic anamorphoses of the kinetic curves of the thermoinactivation of peroxidase immobilized on BrCN-activated Sepharose at 56°C, pH 7.0 (24). Symbols: native, 1; immobilized on BrCN-activated Sepharose, 2; immobilized on albumin-Sepharose, 3; peroxidase-albumin oligomers immobilized on BrCN-activated Sepharose, 4.

support brings about a decrease in stability, as was shown with lipoamide dehydrogenase immobilized on agarose (23).

The chemical nature of the support surface which is in direct contact with an immobilized enzyme markedly affects the thermostability of the immobilized enzyme. Immobilization of peroxidase on BrCN-activated Sepharose (24) sharply decreases the thermostability of the enzyme as compared with the native peroxidase (Fig. 9, curves 1 and 2). In this case the contact of the enzyme with a support does not appear to be favorable. A high increase in the stability of peroxidase has been achieved by two methods:

(1) albumin was covalently attached to Sepharose, the enzyme was immobilized on albumin-Sepharose with the help of glutaraldehyde (Fig. 9, curve 3);

(2) soluble peroxidase-albumin oligomers were obtained, which were then immobilized on BrCN-activated Sepharose (Fig. 9, curve 4). In the latter case the stability of the immobilized enzyme was three orders higher than with peroxidase immobilized without the premodification of the enzyme or support.

High stabilization effects are achieved when carbohydrate residues of peroxidase are used for immobilization (25) (Fig. 10). Peroxidase, in which the carbohydrate groups are oxidized by periodate (Fig. 10, curve 2), does not differ in stability from the native enzyme (Fig. 10, curve 1). After immobilization of the oxidized enzyme on AH-Sepharose a highly stable preparation was obtained (Fig. 10, curve 3). This is apparently a manifestation of the different nature of peroxidase interaction with AH-Sepharose and BrCN-activated Sepharose. The immobilization method produces a considerable effect on the thermostability of enzymes (25). An enzyme can be immobilized on AH-Sepharose via its amino groups, if, for example, glutaraldehyde is used as a cross-linking agent. Two different methods of immobilization were used with peroxidase: (1) the enzyme was

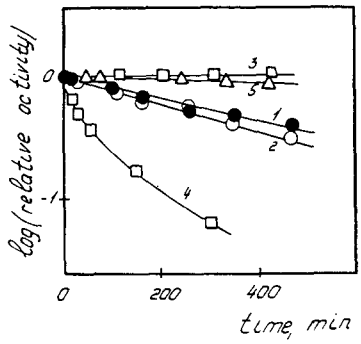

FIG. 10. Semilogarithmic anamorphoses of the kinetic curves of thermoinactivation of peroxidase immobilized on AH-Sepharose at 56°C, pH 7.0 (25). Symbols: native, 1; oxidized by periodic acid, 2; oxidized by periodic acid and then immobilized on AH-Sepharose, 3; modified by glutaraldehyde and then immobilized on AH-Sepharose, 4; immobilized on AH-Sepharose premodified by glutaraldehyde, 5.

modified by dialdehyde and attached to a support (Fig. 10, curve 4; (2) the enzyme was attached to a support modified by dialdehyde (Fig. 10, curve 5). In both cases the activity of the immobilized enzyme was the same, but the stability of the preparations differed hundreds of times. Premodification of a support by dialdehyde results in highly stable preparations, and premodifications of an enzyme by dialdehyde (in solution the stability of such enzyme decreases as compared with the native) yields a low stability immobilized enzyme. This emphasizes once more the role of functional group modification of an enzyme for production of stable derivatives of biocatalysists.

Analysis of the papers dealing with the stabilization of solubles and immobilized enzymes shows that several principles of stabilization have been formulated. One of the interesting approaches was developed in (5): "The more rigid is the structure of the enzyme, the more stable it is." This logically leads to stabilization by multipoint interaction (6), by application of brackets (8), by intrapment into "tight pores" of inert supports (9). All these methods of stabilization aim at fixing the native conformation, at making the unfolding of the enzyme difficult. However, rigidification of the native conformation of the enzymes can be accomplished not "mechanically," but by combining physico-chemical interactions both inside the protein molecule and with its immediate environment in the solution, on the surface of a soluble or insoluble support, or in the pores of a polymeric gel. The highest stabilization effects are achieved with the use of supports on which the native conformation of the enzyme is fixed both by covalent attachment to a support and by noncovalent interaction of the enzyme with the contiguous groups, which gives the maximal values of the free activation energy of enzyme thermoinactivation.

Analysis of thermodynamic activation parameters for thermoinactivation shows that the $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values for the inactivation of modified (soluble or insoluble) enzymes change symbatically in all cases, that is, they

either increase or decrease. Chemical modification of soluble enzymes by low-molecular weight agents leads either to an increase in $\Delta H^{\neq}$ and $\Delta S^{\neq}$, or in other cases, to a decrease. This, undoubtedly, depends on the interplay between the various factors described above. Entrapment of an enzyme into gels or immobilization decreases $\Delta H^{\neq}$ and $\Delta S^{\neq}$ of thermoinactivation in all known cases. This seems to be a regularity. In this case stabilization is provided by the entropy member of $\Delta G^{\neq}$.

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