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CHEMICAL MODIFICATION OF THE ε-AMINO GROUPS OF LYSINE RESIDUES IN HORSERADISH PEROXIDASE AND ITS EFFECT ON THE CATALYTIC PROPERTIES AND THERMOSTABILITY OF THE ENZYME

N.N. UGAROVA, G.D. ROZHKOVA and I.V. BEREZIN

Department of Chemical Enzymology, Lomonosov State University, Moscow, 117234 (U.S.S.R.)

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Summary

Chemical modification of horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) (isoenzyme C) by anhydrides of mono- and dicarboxylic acids and picryl sulfonic acid has been performed. The effect of the modification on the catalytic activity, absorption and circular dichroism spectra of peroxidase has been studied. Rate constants of irreversible thermoinactivation (k_{in}) for the native and modified peroxidase at 56—80° C have been measured. The effective values of the thermodynamic activation parameters of thermoinactivation, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, have been also determined. A relationship between the number of modified ε-amino groups of lysine residues and the nature of the modifier on the one hand, and the conformation and thermostability of the enzyme on the other, is discussed. It has been shown that it is the degree of modification, rather than the nature of the modifier, that produces the major effect on the macromolecular conformation and the thermostability of the enzyme after modification. The conclusion is drawn that the thermostability of the modified enzyme increases due to the decrease of the conformational mobility in the protein moiety around the heme.

Introduction

Chemical modification of the functional groups of enzymes is widely used as a tool for studying the structure and topography of the active centres of

enzymes, localization of individual amino acid residues in a protein globule [1—4] and their participation in the maintenance of the native conformation of a macromolecule [5]. Chemical modification of the functional groups in a protein is necessary for the covalent binding of an enzyme to a support. A number of studies have shown that it is chemical modification that is responsible for the alteration of properties of the enzyme covalently bound to a soluble or an insoluble support [6,7]. ϵ-Amino groups of lysine residues are often used for immobilization of enzymes [8,9]. Therefore the interest in the effect of chemical modification of these groups on the properties, structure and thermostability of soluble enzymes is quite justified. It has been shown [10] that the rate constant of thermoinactivation of an enzyme is a function of the number of modified ϵ-amino groups; however, no systematic study of the effect of the number of modified ϵ-amino groups and the nature of the residue introduced by modification on the structure, the catalytic properties and thermostability of the enzyme has been carried out. This was in fact the purpose of the present investigation in which horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase EC 1.11.1.7) was used as a model enzyme. The peroxidase (isoenzyme C) contains six residues of lysine, the terminal α-amino group being blocked [11]. The enzyme was modified by anhidrides of monocarboxylic acids (acetic, propionic, butyric, valeric, enanthic) and by anhydrides of dicarboxylic acids (succinic and maleic) and by picryl sulfonic acid. For the native and modified peroxidase, the catalytic activity, ultraviolet and CD spectra, and temperature dependences of thermoinactivation constants were determined.

Experimental

Materials

Horseradish peroxidase (isoenzyme C by the Shannon classification [12]), was isolated from a commercial preparation of Reanal, in a homogeneous state $RZ = A_{403}/A_{278} = 3.3$, as described in Ref. 13. In a number of experiments a peroxidase of Sigma, type VI, was used. For both preparations ultraviolet and CD spectra, the catalytic activity, the content of lysine residues and the temperature dependences of thermoinactivation constants were practically identical. The commercial carboxylic acid anhydrides (acetic, propionic, butyric, valeric, enanthic, maleic) were distilled twice in vacuum before use. The sodium dodecyl sulfate (Reanal) was crystallized twice from alcohol. *o*-Dianisidine was sublimed twice in vacuum. Picryl sulfonic acid was purchased from Sigma. All other chemicals used were of analytical grade. The water was distilled trice.

Methods

The concentration of hemin in the native or modified peroxidase was determined by the pyridine hemochromogen method [14].

Catalytic activity of the peroxidase was assayed at 20° C by measuring the initial rate of oxidation of *o*-dianisidine by H_2O_2 using the two substrates in saturating concentrations [15,16]. To 2.3 ml of 0.01 M sodium phosphate buffer (0.1 M KNO_3, pH 7.0) there were added 0.07 ml 5.7 mM *o*-dianisidine in

96% ethanol, 0.07 ml 21 mM hydrogen peroxide and 0.05 ml of the 0.1—1.5 nM enzyme solution. The increase in the absorbance at 460 nm after intensive stirring of the mixture (ϵ_{460} = 30 000 $M^{-1} \cdot cm^{-1}$ for the product of the oxidation [16]) was recorded by a B2-25 Beckman spectrophotometer. A quantity of *o*-dianisidine (μmol) oxidised per s by the quantity of the native or modified enzyme which contained 1 μmol of hemin was assumed to be a measure of the enzyme activity. The enzyme activity of the peroxidase at elevated temperatures was assayed as shown above. Before the last component (H_2O_2) was added the reaction mixture had been incubated in a thermostatically controlled spectrophotometric cuvette.

Determination of the number of free ϵ-amino groups of lysine residues in the native and modified peroxidase was carried out by titrating the enzyme by picryl sulfonic acid as suggested for cytochrome C [17] and described in detail in [18].

Modification of the enzyme by picryl sulfonic acid. 1.5 mg of the enzyme were dissolved in 0.2 ml of 1% picryl sulfonic acid in 2% $NaHCO_3$, pH 8.0 and incubated for 2 h at 0°C (or at 40°C). After that the solution was passed through a 1 × 30 cm column packed with Sephadex G-25 (Pharmacia, Sweden) in 0.05 M NaCl. It was found that picryl sulfonic acid at 0°C modified three ϵ-amino groups, and at 40°C all the six ϵ-amino groups in a molecule of the peroxidase.

Modification of the peroxidase by anhydrides of carboxylic acids was carried out as described in [19]. To 3 ml of 50 μM peroxidase in 0.01 M sodium phosphate buffer (0.1 M KNO_3, pH 8.0) at 0°C 90 μmol anhydride were added for 1 h in small portions with energetic stirring. The pH was maintained at 8.0 by addition of 5 M NaOH. This enzyme solution was dialyzed against water for 24 h at 4°C and lyophilized. The degree of modification was evaluated by titration of free ϵ-amino groups by picryl sulfonic acid [18]. It was shown that at a 50—100-fold excess of the anhydrides with respect to the number of ϵ-amino groups of peroxidase, four ϵ-amino groups of peroxidase were modified by all anhydrides studied. With enanthic anhydride we accomplished modification of two and three ϵ-amino groups using a two- or five-fold excess of the anhydride.

Using the alkaline hydroxylamine reaction [20] we found that all anhydrides studied modify a great number of OH groups of peroxidase. The cleavage of these esters was accomplished with hydroxylamine at pH 12 for 2 min at 25°C in order to obtain a derivative limited to amino substitution only [20]. Identity of ultraviolet and CD spectra, activity and thermostability of these derivatives and peroxidase modified by anhydrides has been observed. It proves that acylation of OH-groups of peroxidase does not notably affect the studied properties of the enzyme. So none of the preparations described below was treated by hydroxylamine.

Absorption and circular dichroism spectra of the native and modified peroxidase were recorded using the enzyme solutions in 0.05 M NaCl. The absorption spectra were recorded by a B2-25 Beckman spectrophotometer. CD spectra were recorded by a Dichrograph Mark-III, JOBIN-IVON.

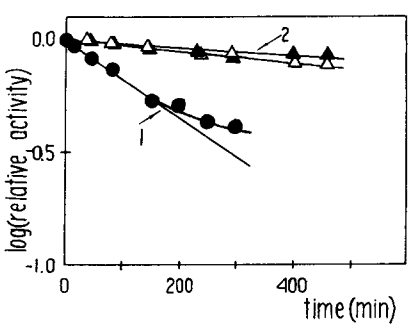
Thermoinactivation of the native and modified peroxidase was carried out as described in Ref. 13. A 0.1 μM solution of the enzyme (pH 7.0, 0.01 M sodium phosphate buffer, 0.1 M KNO_3) was incubated at a controlled temperature.

0.05 ml aliquots were taken at certain time intervals and put into 10 ml buffer solution at 20° C to provide fast cooling. The residual catalytic activity was determined in the fashion described above. It was found that the kinetics of thermoinactivation do not depend on the starting concentration of the native or modified peroxidase (0.05—1 μM). This means that the observed inactivation of the enzyme is a monomolecular process. The catalytic activity of the enzyme versus the duration of the incubation at a given temperature is represented graphically by a straight line up to a 50% degree of inactivation (semilogarithmic scale) (Fig. 1). The first-order constant, k_{in}, was measured from the initial part of the inactivation slope at a given temperature.

Results and Discussion

Accessibility of the ε-amino groups of lysine residues in the peroxidase to modifiers

At 100-fold excess of the modifiers with respect to amount of ε-amino groups of the enzyme anhydrides of mono- and dicarboxylic acids modify four ε-amino groups in the peroxidase for 1 h at 0° C (pH 8.0). The number of modified ε-amino groups does not depend on the length of a hydrocarbon residue in the anhydride molecule (in a series of acetic-enanthic anhydrides) or on whether the given agent is an anhydride of a mono- or dicarboxylic acid. At 0° C for 2 h picryl sulfonic acid modifies only three amino groups. At 40° C under the same conditions it modifies all the six amino groups of the peroxidase. As it has been shown in [21] at 22° C, pH 9.5 picryl sulfonic acid modified four amino groups of peroxidase. Anhydrides of mono- and dicarboxylic acids are highly reactive compounds and in the conditions used complete acylation of ε-amino groups proceeds [19]. Picryl sulfonic acid is an agent of moderate activity with respect to amino groups [1]. However, with its



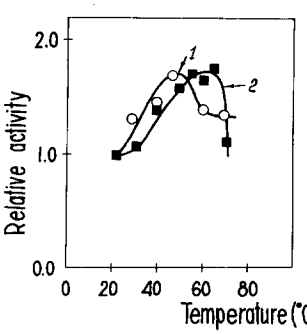


Fig. 1. Kinetic curves of thermoinactivation for the native and modified peroxidase in semilogarithmic coordinates. Conditions of thermoinactivation: 60° C, pH 7.0 (0.01 M sodium phosphate buffer, 0.1 M KNO_3), 0.1 μM peroxidase. Conditions of activity assay: 0.01 M sodium phosphate buffer, 0.1 M KNO_3, pH 7.0, 20° C, 0.16 mM *o*-dianisidine, 0.6 mM hydrogen peroxide, 0.1—1.0 nM peroxidase; 1, native enzyme; 2, enzyme modified by acetic anhydride (△) and then treated by hydroxylamine to cleave *O*-acyl esters (▲).

Fig. 2. Temperature dependence of the catalytic activity of the native and modified peroxidase. Conditions are the same as in Fig. 1. 1, native peroxidase; 2, peroxidase where four amino groups have been modified by enanthic anhydride.

concentration and incubation time used, the reaction of the amino groups with picryl sulfonic acid is always complete [21]. The fact that picryl sulfonic acid and anhydrides at 0°C modify various numbers of amino groups may be explained rather by different accessibility of amino groups than by different activity of the modifiers. On the other hand, various accessibility of the ϵ-amino groups to the picryl sulfonic acid at 0, 22 and 40°C may be the result of a 'loosening' of the macromolecule when the temperature is elevated.

Catalytic properties of the modified peroxidase

The modified peroxidase regardless the degree of modification and the nature of the modifier maintains its complete catalytic activity at pH 7.0, 20°C. This fact supports the idea that the modification studied does not effect on the active centre of the enzyme.

Fig. 2 shows the temperature dependences of the catalytic activity of the native and enanthic anhydride-modified peroxidase. For the peroxidase modified by different anhydrides, the temperature dependences curve is similar to the one shown in Fig. 2 curve 2. The native enzyme has the highest catalytic activity around 50°C, whereas the modified enzyme displays a maximum activity at 55—65°C. The catalytic activity of the modified enzyme rapidly decreases above 65°C, contrary to the behaviour of the native enzyme. These changes in the temperature dependence of the catalytic activity may be the result of the modification-induced changes in the physicochemical properties of the enzyme [23].

The changes in the structure of the modified peroxidase revealed from the absorption, fluorescence and circular dichroism spectra

Absorption and CD spectra for the native peroxidase and that modified by acetic, butyric, valeric, enanthic and succinic anhydrides and picryl sulfonic acid have been measured. It is found that the spectra of peroxidase modified by the various anhydrides of monocarboxylic acids closely resemble one another (within the experimental error limits). Therefore Figs. 3 and 4 show the spectra for only one of the preparations of peroxidase modified by a monocarboxylic acid anhydride (butyric anhydride). There is little difference between absorption spectra of the native peroxidase and those of the enzyme modified by the various anhydrides (Figs. 3 and 4). Small differences in the region of 260—280 nm and 360—390 nm and in the visible region are more pronounced for the succinylated peroxidase. Picryl sulfonic acid modification naturally induces great changes in the absorption spectra as a result of the presence of trinitrophenyl chromophores in the modified enzyme.

The CD spectrum of the native peroxidase is similar to that described in Ref. 24. For native and modified enzyme, CD spectra are substantially different. The peroxidase with three and four modified amino groups shows the pronounced increase in the intensity of the heme band at 549 nm, and decrease in the intensity in the 260—300 nm region compared to the native enzyme. For peroxidase with six modified ϵ-amino groups the decrease in the intensity at 549 nm compared to the native enzyme is observed. For the picryl sulfonic acid-modified peroxidase the changes in CD spectra in the 260—400 nm region are the result of the trinitrophenyl chromophores being placed into assym-

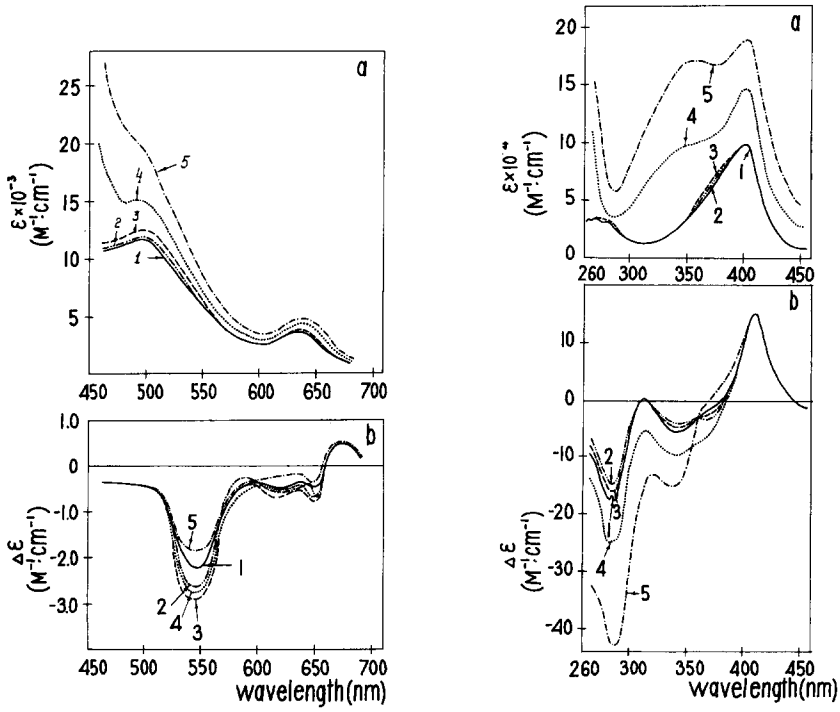


Fig. 3. Absorption (a) and circular dichroism (b) spectra of the native and modified peroxidase. Conditions: enzyme solution in 0.05 M NaCl, 20°C. 1, native peroxidase, (———); peroxidase modified: 2, by butyric anhydride (· · —· · —); 3, by succinic anhydride (- - - - -); 4, 5, three (· · · · · ·) and six amino groups (· — · — ·) are modified by picryl sulfonic acid.

Fig. 4. Absorption (a) and circular dichroism (b) spectra of the native and modified peroxidase. Conditions and denotation of curves as in Fig. 3.

metrical environment after binding to the protein. In the 260—350 nm region there is proportionality between the intensity and the number of trinitrophenyl groups binding to the enzyme, but in the 360—400 nm region this proportionality does not appear. Different conformation of the heme environment in these two preparations may produce this change. The increased intensity of the heme bands in CD spectra implies that the conformational mobility has decreased and the protein around the heme has become more rigid. This is observed on chemical modification of three or four amino groups in the peroxidase. On the other hand, modification of six amino groups by picryl sulfonic acid decreases rigidity of the protein around the heme. This is a result of conformational changes which 'loosen' the protein around the heme.

The pH-dependence of the fluorescence intensity is an important physicochemical characteristic of proteins. The higher fluorescence intensity of the tryptophan residue in the peroxidase at pH <3.0 is a result of acid denaturation of the enzyme [25]. As pH-dependence of the fluorescence shows (Fig. 5), for the peroxidase with four ϵ-amino groups modified by enanthic anhydride, denaturation starts at more acid pH (pH <2.0). This is an indication of the difference in the conformations of the native and enanthic anhydride modified peroxidases.

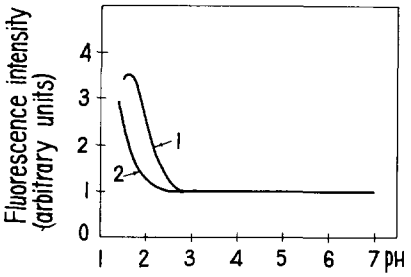


Fig. 5. pH-dependence of fluorescence intensity for the native (1) and modified by enanthic anhydride (2) peroxidase. Conditions: 0.5 μM of peroxidase in 5 mM Na_2HPO_4, 20°C, titrated by 1 M HCl. Excitation by 290 nm light. Fluorescence was measured at 340 nm.

Temperature dependence of the rate constants of irreversible thermoinactivation for the native and modified peroxidase

The rate constants of irreversible thermoinactivation for the native and modified peroxidase (k_{in}) were measured in the 56 to 80°C temperature range. From the log k_{in} vs. inverse temperature, $1/T$, curves (Fig. 6), effective values of the thermodynamic activation parameters, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, were derived (Table I). The Arrhenius plots for the native and modified peroxidase intersect at 75 ± 3°C (isokinetic temperature). For the majority of the preparations of

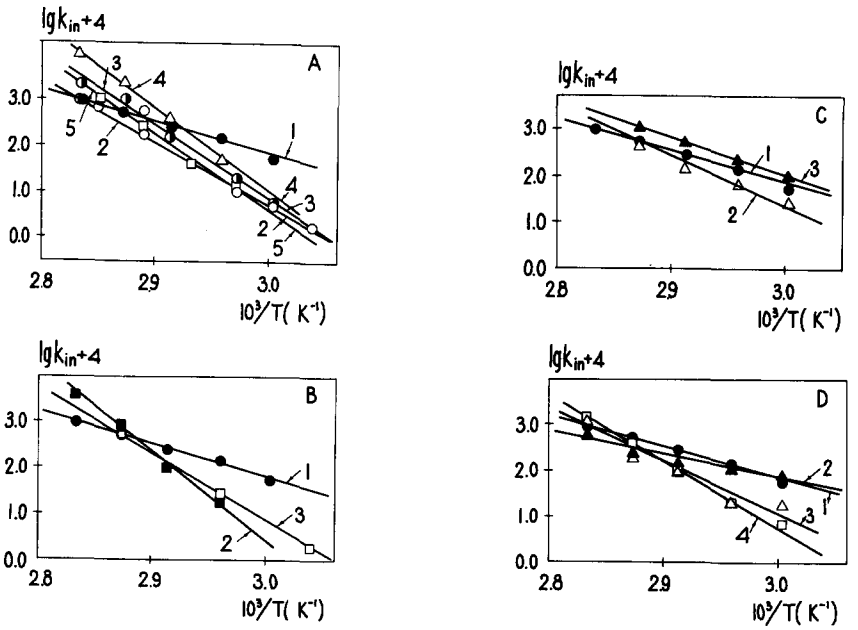


Fig. 6. Temperature dependence of the first-order rate constant of monomolecular thermoinactivation (log k_{in}) for the native and modified peroxidase. Conditions are the same as in Fig. 1. A. 1, native peroxidase; 2—5, peroxidase modified by acetic (2), propionic (3), butyric (4), valeric (5) anhydrides. B. 1, native peroxidase; 2, 3, peroxidase modified by succinic (2) and maleic (3) anhydrides. C. 1, native peroxidase; 2, 3, peroxidase which has three (2) and six (3) amino groups modified by picryl sulfonic acid. D. 1, native peroxidase; 2, 3, 4, peroxidase which has two (2), three (3) and four (4) amino groups modified by enanthic anhydride.

TABLE I

THERMODYNAMIC ACTIVATION PARAMETERS OF IRREVERSIBLE THERMOINACTIVATION OF THE NATIVE AND MODIFIED PEROXIDASE IN THE 56° TO 80°C TEMPERATURE RANGE

Conditions of inactivation: 0.01 M sodium phosphate buffer, 0.1 M KNO_3, pH 7.0, 0.1 μM enzyme. Conditions of activity assay: 0.01 M sodium phosphate buffer, 0.1 M KNO_3, pH 7.0, 20°C, 0.16 mM *o*-dianisidine, 0.6 mM hydrogen peroxide, 0.1—1.0 nM peroxidase.

Sample	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal/deg. per mol)	t isokinet. (°C)	Stabilization relative to native enzyme at 56°C
Native peroxidase	31 ± 1	14 ± 3	—	1.0 *
Peroxidase modified by anhydrides E-$(COR)_x$, where R:				
$-CH_3$	64 ± 1	110 ± 6	80	32
$-C_2H_5$	71 ± 4	133 ± 6	74	23
$-C_3H_7$	79 ± 1	158 ± 4	69	20
$-C_4H_9$	73 ± 5	136 ± 6	75	45
$-C_6H_{13}$	65 ± 2	114 ± 5	76	18
$-C_2H_4COOH$	87 ± 1	178 ± 5	73	90
$-CH=CH-COOH$	68 ± 1	123 ± 6	75	26
Peroxidase modified by enanthic anhydride E-$(NHCOC_6H_{13})_m$,				
where m = 2	25 ± 3	1 ± 6	64	0.8
3	50 ± 5	71 ± 6	75	7
4	65 ± 2	114 ± 5	76	18
Peroxidase modified by picryl sulfonic acid E-$[NHC_6H_2(NO_2)_3]_m$				
where m = 3	45 ± 2	57 ± 4	75	3
6	33 ± 2	24 ± 3	41	0.7

* k_{in} of the native peroxidase at 56°C is $(5.5 \pm 0.3) \cdot 10^{-3}$ min^{-1}.

the modified enzyme, the thermostability at the temperature below the isokinetic was much greater than that of the native enzyme due to the higher value of activation enthalpy, $\Delta H^{\neq}$. The lower the temperature, the greater the effect of stabilization for the modified enzyme. For example, the thermostability of the modified peroxidase at 56°C is 20—90-fold greater than that of the native one (Table I). This is an example of the so-called 'low-temperature' stabilization of enzymes. It should be noted that immobilization of enzymes usually decreases $\Delta H^{\neq}$ of thermoinactivation compared to the native enzymes [26—29]. In this case a higher thermostability is obtained at the temperatures which are higher than the isokinetic ('high-temperature' stabilization).

The activation entropy, $\Delta S^{\neq}$, of thermoinactivation changes in the same direction as $\Delta H^{\neq}$ (Table I). An experimental compensation relationship [30, 31] between $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for thermoinactivation of the native and modified peroxidase (Fig. 7) demonstrates that, depending on the degree of modification and the nature of the modifier, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ can vary significantly, whereas the variation range of activation free energy, $\Delta G^{\neq}$, is only 2—3 kcal/mol (Table I). We shall discuss below the effect of modification on $\Delta H^{\neq}$ and $\Delta S^{\neq}$ of thermoinactivation, instead of comparing rate constants of inactivation at one temperature, as is usually the case. The rate constant of thermoinactivation, k_{in}, depends on temperature and, therefore, the effect of stabilization, i.e., the

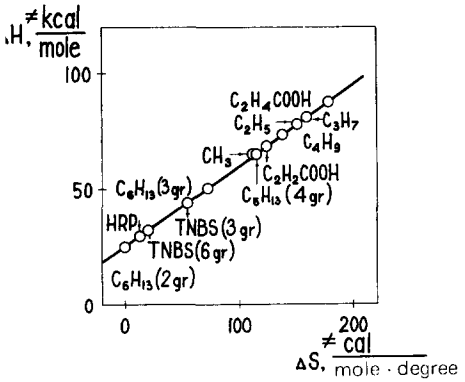


Fig. 7. Experimental compensation relationship between $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the process of irreversible thermoinactivation of the native and modified peroxidase. In brackets, the number of the residues introduced into the molecule of the enzyme during modification. Abbreviations: HRP, peroxidase; TNBS, picryl sulfonic acid.

k_{in} ratio for the native and modified enzyme, is a function of temperature. At the same time, the thermodynamic inactivation parameters, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ can be regarded over a sufficiently narrow temperature interval, as being temperature-independent, and these values are, in our opinion, a more objective criterion for characterization of the effect of chemical modification on the thermostability of the enzyme. It should be emphasized that at temperature lower than the isokinetic one, the thermostability of the modified, compared to the native, enzyme will be the higher, the higher is the respective $\Delta H^{\neq}$ value.

The effect of the degree of modification on thermostability of modified peroxidase

The effect of the number of modified amino groups (m) on the thermostability of peroxidase has been studied with (i) peroxidase modified by enanthic anhydride (with m = 2, 3 and 4), and (ii) peroxidase modified with picryl sulfonic acid (m = 3 and 6). We failed to carry out modification of all the six amino groups (from 1 to 6) by the same modifier, which, of course, makes our conclusions somewhat incomplete. Nevertheless, as shown by the results in Table I and Figs. 6C and 6D, the number of modified amino groups produces the main effect on the $\Delta H^{\neq}$ and $\Delta S^{\neq}$ of thermoinactivation. For peroxidase modified by enanthic anhydride, with m = 2 the $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values were somewhat lower than those of the native enzyme. As m increases from 2 to 4, an almost proportional increase in both $\Delta H^{\neq}$ and $\Delta S^{\neq}$ is observed. For peroxidase modified by picryl sulfonic acid with m = 3, the values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ are similar to those for peroxidase modified by enanthic anhydride also with m = 3 (Fig. 8). Modification of the six amino groups of peroxidase by picryl sulfonic acid (m = 6) decreases $\Delta H^{\neq}$ and $\Delta S^{\neq}$ compared to the case with m = 3. Thus with m = 3, regardless of the nature of the modifier, $\Delta H^{\neq}$ is 45—46 kcal/mol and $\Delta S^{\neq}$ equals 57—71 kcal/degree per mol. As seen from Table I, for all the anhydrides studied, with m = 4, $\Delta H^{\neq}$ lies between 64 and 87 kcal/mol, and $\Delta S^{\neq}$ between 110 and 178 kcal/degree per mol. For various m values, the ranges $\Delta H^{\neq}$ and $\Delta S^{\neq}$ do not overlap, although the nature of residue R bound to

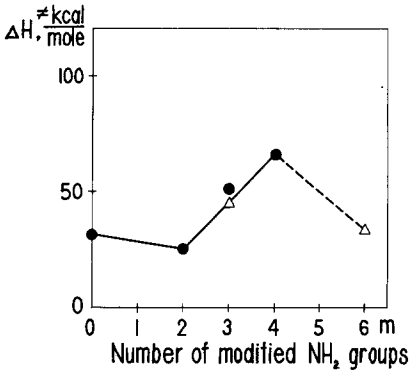


Fig. 8. The effect of the number of modified amino groups in the peroxidase on the value of activation enthalpy, $\Delta H^{\neq}$, for irreversible thermoinactivation of the enzyme. Circles indicate data for the peroxidase modified by enanthic anhydride, triangles for the peroxidase modified by picryl sulfonic acid.

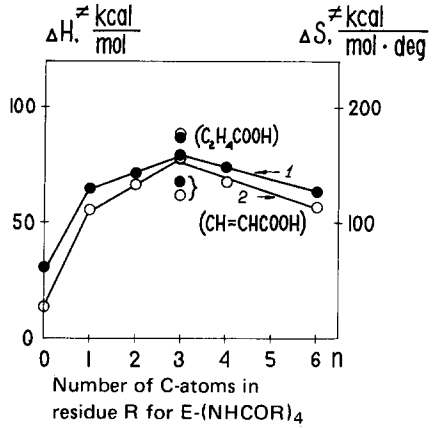


Fig. 9. Effective values of thermodynamic activation parameters, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, for irreversible thermoinactivation of the native peroxidase and that modified by anhydrides of carboxylic acids in relation to the length (n) of a hydrocarbon residue R introduced into the enzyme via modification. 1, $\Delta H^{\neq}$ change; 2, $\Delta S^{\neq}$ change.

the enzyme considerably changes. This allows the suggestion to be made that it is the degree of modification that mostly affects the thermostability of the enzyme. The same conclusion was made by the authors who studied the thermostability of α-chymotrypsin in relation to the number of amino groups modified by acrolein [10].

The effect of the chemical nature of a modifier on the thermostability of modified peroxidase

For peroxidase with four modified amino groups, the effect of the structure and charge of residue R on the thermostability of modified enzyme was studied. Modification of four amino groups, charged positively in neutral solutions by anhydrides of monocarboxylic acids changes the net charge of the molecule, its conformation (see above) and thermostability (Table I and Fig. 6). The thermostability of the enzyme was studied at pH 7.0, where the globule of peroxidase is charged positively (pI = 8.8 for the isoenzyme C [12]), and the decrease in the positive net charge can indeed stabilize the macromolecule. It should be noted, however, that within the pH 5—9 range the thermostability of the enzyme is pH-independent [13]. We have also ascertained that the thermostability of peroxidase modified by propionic, butyric and succinic anhydrides remains constant within the pH 5—9 range. There are grounds for believing, therefore, that in this case the change in the conformation and thermostability of the enzyme is associated not only, or not so much, with the decrease in the positive net charge but also or rather, with the decrease in the local charge in the region of the macromolecule that is essential for stabilization of the native structure of the enzyme. At the same time, the acylation of non-charged OH-groups does not affect the conformation and thermostability of peroxidase, as has been shown above. Thus, the change

of conformation and thermostability of the modified enzyme may be achieved mainly by modification of the charged functional groups of protein.

The structure of radical R is of secondary importance for the thermostability of the modified enzyme (Table I, Fig. 6A). It was of interest, nevertheless, to estimate the contribution of the hydrophobicity of residue R into the altered thermostability of acylated peroxidase. To this end, a series of acylated peroxidases, E-$(COR)_x$ was studied, where R was varied from $-CH_3$ to $-C_6H_{13}$. We compared only the members of this series, rather than the native and modified enzyme, and analyzed changes in $\Delta H^{\neq}$ and $\Delta S^{\neq}$ in relation to the number of carbon atoms (n) in residue R. Each member of the series differs from the preceding one by one methylene group. An increase in the number of carbon atoms (n) in residue R from 1 to 3 makes $\Delta H^{\neq}$ and $\Delta S^{\neq}$ greater. Further increase in n makes $\Delta H^{\neq}$ and $\Delta S^{\neq}$ lower by the same value (Fig. 9). The optimum length of residue R for the acylated peroxidase, as is seen from Fig. 9, is three. The increase of thermostability of proteins in the course of their noncovalent interaction with hydrocarbons was observed for several proteins [32] since hydrophobic interactions play the main role in the stabilization of the tertiary structure of proteins [33]. If the hydrocarbon residue is of the same length, a negatively charged group to be introduced into the enzyme significantly increases $\Delta H^{\neq}$. The peroxidases modified by butyric and succinic anhydrides have $\Delta H^{\neq}$ values that differ by about 10 kcal/mol. Lower values of $\Delta H^{\neq}$ for the peroxidase modified by maleic anhydride could be explained by unfavorable interactions between the protein and a maleic acid residue which has a rigid double bond. Therefore, not only the length of residue R (its hydrophobicity) but also the charge on R affect $\Delta H^{\neq}$ of thermoinactivation of the modified peroxidase.

Relationship between the modification-induced structure changes and thermostability of the enzyme

A study of CD spectra (Fig. 3 and 4) shows that the peroxidase with four modified amino groups has a restricted conformational mobility of the protein in the vicinity of the heme. At the same time, thermostability of the modified enzyme was significantly increased (at temperatures below the isokinetic). Modification of all the six amino groups of the enzyme reduces the rigidity of the protein in the vicinity of the heme and the thermostability of the enzyme (Fig. 6C). This means that there is a distinct correlation between the conformation changes and the change of the thermostability of the enzyme after modification of its functional groups. It must be noted that the $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values of thermoinactivation happen to be more sensitive to the degree of modification and the nature of the modifier than do CD spectra. Although the $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values are effective, an analysis of their variations is a very powerful tool for studying the conformation-function relationship of marcomolecules, as well as the effect of chemical modification on the conformation and thermostability of proteins.

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References

1 Shibata, K. (1971) in New Techniques in amino acid, peptide and protein analysis (Niederwieser, A. and Pataka, G., eds.),
2 Klippenstein, G.L. (1972) Biochem. Biophys. Res. Commun. 49, 1474—1479
3 Shi, J.H.C., Shannon, L.M., Kay E. and Lew, J.Y. (1971) J. Biol. Chem. 246, 4546—4551
4 Huntley, T.E. and Strittmatter, P. (1972) J. Biol. Chem. 247, 4648—4653
5 Myer, Y.P. (1972) Biochemistry 11, 4203—4208
6 Larionova, N.I., Kazanskaya, N.F. and Sacharov, I.Yu. (1977) Biochimia (Russ.) 49, 1237—1243
7 Ugarova, N.N., Brovko, L.Yu., Rozhkova, G.D. and Berezin, I.V. (1977) Biochemistry (Russ.) 42, 943—949
8 Zaborsky, O.R. (1973) Immobilized Enzymes, C.R.C. Press, Cleveland, OH
9 (1976) Immobilized Enzymes (Berezin, I.V., Antonov, V.K. and Martinek, K., eds.), Vol. I, Lomonosov State University Press, Moscow
10 Torchilin, V.P., Maksimenko, A.V., Smirnov, V.N., Klibanov, A.M. and Martinek, K. (1979) Biochim. Biophys. Acta 567, 1—11
11 Welinder, K.G. (1976) FEBS Lett. 72, 19—23
12 Shannon, L.M., Kay, E. and Lew, J.Y. (1966) J. Biol. Chem. 241, 2166—2172
13 Berezin, I.V., Ugarova, N.N., Kershengoltz, B.M. and Brovko, L.Yu. (1975) Biochimia (Russ.) 40, 297—301
14 Falk, J.E. (1964) Porphyrins and Metalloporphyrins, p. 184, Elsevier, Amsterdam
15 Berezin, I.V., Ugarova, N.N. and Kershengoltz, B.M. (1974) Dokl. Akad. Nauk. S.S.S.R. 214, 701—704
16 Lebedeva, O.V., Ugarova, N.N. and Berezin, I.V. (1977) Biochimia (Russ.) 42, 1372—1379
17 Pettigrew, G.W., Aviram, I. and Schejter, A. (1976) Biochim. Biophys. Res. Commun. 807—813
18 Ugarova, N.N., Rozhkova, G.D., Vasilieva, T.E. and Berezin, I.V. (1978) Biochimia (Russ.) 43, 793—797
19 Habeeb, A.F.S.A., Cassidy, H.G. and Singer, S.J. (1958) Biochim. Biophys. Acta 29, 587—593
20 Gounaris, A.D. and Perlmann, G.E. (1967) J. Biol. Chem. 242, 2739—2745
21 Kershengoltz, B.M., Ugarova, N.N. and Berezin, I.V. (1976) Bioorg. Chem. (Russ.) 2, 264—272
22 Fields, R. (1971) Biochem. J. 124, 581—590
23 Alexandrov, V.Ya. (1975) Cells, Macromolecules and Temperature, Izd. Nauka, Leningrad (Russ.)
24 Strikland, E.N., Kay, E., Shannon, L.M. and Hotwitz, I. (1968) J. Biol. Chem. 243, 3560—3565
25 Savitskii, A.P., Ugarova, N.N. and Berezin, I.V. (1977) Bioorg. Chem. (Russ.) 3, 1242—1250
26 Martinek, K., Klibanov, A.M., Goldmacher, V.S. and Berezin, I.V. (1977) Biochim. Biophys. Acta 485, 1—12
27 Kinstler, O.B. and Kozlov, L.V. (1977) Biochimia (Russ.) 42, 1674—1681
28 Baeva, V.S., Kozlov, L.V., Antonov, V.K. and Tichomirova, A.S. (1977) Biochimia 42, 706—711
29 Kaboli, H. and Reilly, P.J. (1978) in Proc. of the third joint U.S./U.S.S.R. Enzyme Eng. Seminar held at Tallin, U.S.S.R. (Bungay, H.R., ed.), pp. 102—123, NSF, Washington
30 Lumry, R. and Biltonen, R. (1969) in Structure and Stability of Biological Macromolecules (Timasheff, S.N. and Fasman, G.D., eds.), pp. 65—212, Marcel Dekker, New York
31 Lumry, R. and Rajender, S. (1970) Biopolymers, 9, 1125—1227
32 Izmailova, V.N. and Rebinder, P.A. (1974) Structure Formation in Protein Systems, p. 28, Izd. Nauka, M. (Russ.)
33 Jencks, W.P. (1969) Catalysis in Chemistry and Enzymology, Chapter 8, McGraw-Hill, New York