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Chemical Approaches to Intermediates of Enzyme Catalysis

By PH. CHRISTEN*

Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston (Massachusetts, USA)

The understanding of the molecular basis of enzyme action will depend ultimately on the elucidation of the structural and chemical properties of both the enzyme and its substrate complexes. A wide assortment of approaches has been developed and applied to the study of enzymes, including determination of their three-dimensional structure by X-ray crystallography and synthesis of proteins with specific enzymatic activity[1,2]. However, the exploration of the dynamic features of enzyme catalysis, i.e. of enzyme-substrate intermediates, has not proceeded to a comparable extent due to lack of effective experimental means. The present study discusses a novel approach to the chemistry of enzymatic reaction intermediates. Chemical probing with suitable reagents of catalytically active enzyme-substrate complexes appears to have promise as a method for the elucidation of the detailed features of enzymatic reactions.

Introduction

Controlled chemical modification has long served as a major tool in relating protein structure to the catalytic function of enzymes and both experimental techniques and their intellectual foundations have been refined greatly[3–6]. A significant advance was achieved by the recognition that active site residues often exhibit chemical properties which are unusual when compared with those of other residues of the protein. In many cases the exceptionally high reactivity of active site residues allows their selective modification with a reagent which – of itself – is not specific. These conspicuous chemical properties can be demonstrated already in the absence of substrate, and apparently arise from the environment generated by the juxtaposition of various amino acid side chains consequent to polypeptide folding. The specific chemistry of functional groups appears to be closely related to their role in catalysis. Both the unusual reactivity and enzymatic function are lost when the protein is denatured.

Chemical modifications of functional groups of enzymes have been carried out mostly in the absence of substrate. Generally, it is assumed that the presence of substrate would prevent reaction of critical residues at the active site. In fact, protection by substrate against inactivation by chemical modification is often taken as a criterion for locating a residue critical to activity. However, a novel perspective of chemical modifications in the presence of substrate is indicated by recent observations which suggest that the occurrence of metastable intermediates in reaction mechanisms might be a fundamental feature of enzyme catalysis[7–9]. The conformational flexibility of enzyme macromolecules[10] might make it feasible to divide the enzymatic reaction into a number of discrete steps by alteration of the topochemistry of active sites in the course of catalysis. Reactions might proceed through a series of intermediate complexes, any one of which might exhibit specific chemical reactivities.

One approach to identifying catalysis-linked changes in the chemical behavior of enzyme-substrate complexes would be direct examination of the reactivity of intermediates toward suitable reagents. In the present study ternary systems consisting of enzyme, substrate, and a probing reagent were examined for catalysis-dependent reactions, i.e. occurring only in the complete system and not observed with the reagent and enzyme or substrate (and product) alone. According to the action-reaction principle, catalysis-induced reactivity ought to be detectable in the enzyme as well as in the substrate moiety of intermediate complexes. We have used tetranitromethane (TNM), a reagent that seemed suitable for examination of both cases; under conditions appropriate for enzyme activity,

* Present address: Biochem. Institut der Universität, CH-8032 Zürich (Switzerland).

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TNM can nitrate tyrosyl residues[11,12], oxidize sulfhydryl groups[13], and react readily with carbanions[14], which are intermediates in a number of enzymatic reactions[15,16].

The TNM-carbanion reaction was applied to yeast and muscle aldolase, where an intermediate carbanion on the substrate moiety of the enzyme-substrate complex could be detected. Further, catalysis-induced reactivity of an enzyme side chain could be demonstrated with aspartate aminotransferase. An essential tyrosyl residue, unreactive in the native enzyme, becomes unusually reactive during the process of transamination, allowing its selective nitration.

Thus, chemical probing appears to be a feasible method for examining enzyme-substrate intermediates and the information which the method already has produced illustrates the great potential of this heretofore neglected approach to enzyme catalysis.

Experimental

1. *The probing reagent: tetranitromethane.* A suitable probing reagent should react specifically either with a particular reaction intermediate, e.g. a carbanion, or with a particular type of amino acid residue. Catalysis-induced reactivity is detected by comparison of the reaction of the probing reagent with an enzyme-substrate system and with enzyme or substrate (and product) alone. The reagent should react under conditions appropriate for catalysis and its reaction should be readily quantitated. TNM has been found to be suitable for reaction with carbanions and for modification of protein side chains. The pertinent experimental procedures are outlined below.

2. *Chemical probing of the substrate moiety; the TNM-carbanion reaction.* TNM has been shown to react readily with carbanions, permitting, e.g. titration of the anionic form of carbon acids[14]. The reaction can be followed spectrophotometrically by virtue of the intense absorbance of the yellow by-product nitroformate, $\varepsilon_{350} = 14{,}400$[17]. Based on these features chemical probing of carbanionic enzyme-substrate intermediates with TNM[18–20] may be represented by equation 1:

$$\begin{array}{c} E + S \rightleftarrows E - S \rightleftarrows E - S^{\ominus} \rightleftarrows E - P \rightleftarrows E + P \\ \downarrow + \text{TNM} \\ E - [SNO_2] + C(NO_2)_3^{\ominus} \\ \downarrow \\ E + [SNO_2] \end{array} \qquad (1)$$

E denotes the enzyme, S the substrate, and P the product(s). In the presence of TNM, the carbanionic enzyme substrate intermediate, E–S$^{\ominus}$, is partitioned between the usual enzymatic pathway and the trapping reaction with TNM leading to an enzyme-modified substrate complex, E–[SNO_2], which subsequently dissociates to unaltered enzyme and modified substrate. Since only a small fraction of the reactive intermediate appears to interact with TNM, the rate of nitroformate production provides a convenient gauge of the relative concentration or reactivity of the carbanion intermediate under different conditions. The rates of nitroformate production in enzyme-substrate systems reported here are initial rates and were determined by obtaining the slope of the tangent to the absorbance (A_{350}) vs. time curve 6–8 sec after addition of the enzyme. All rates are corrected for nitroformate production induced by the enzyme alone in the absence of substrate.

3. *Chemical probing of the enzyme moiety; catalysis-dependent nitration of a functional tyrosyl residue.* The principle of catalysis-dependent modification of the enzyme moiety of enzyme-substrate intermediates is illustrated in equation 2.

$$\begin{array}{c} E + S \rightleftarrows E - S \rightleftarrows E \overset{*}{-} S \rightleftarrows E - P \rightleftarrows E + P \\ \downarrow + \text{TNM} \\ E \cdot NO_2 + S + C(NO_2)_3^{\ominus} \end{array} \qquad (2)$$

E$\overset{*}{-}$S denotes a particular enzyme-substrate intermediate distinguished by an unusually reactive side chain of the enzyme protein. In the case of aspartate aminotransferase[21,22] this catalysis-activated side chain is a tyrosyl residue which reacts with TNM to yield a nitrated enzyme, E·NO_2. Nitration of tyrosyl residues by TNM has been previously demonstrated with a number of peptides and proteins. The reaction apparently involves the phenoxide ion and yields 3-nitrotyrosine. The absorbance of this product ($\varepsilon_{428}^{pH\,8.5} = 4100$) can be employed for quantitation of the degree of nitration[12].

Results and discussion

A. *Chemical probing of the substrate moiety; carbanionic aldolase-substrate intermediates*

1. *Aldolase from rabbit muscle.* The reaction of TNM with a carbanionic reaction intermediate of aldolase from muscle[18,19] will serve to illustrate chemical probing of the substrate moiety of an enzyme-substrate complex.

Fructose 1,6-diphosphate aldolase from rabbit muscle catalyzes the reversible cleavage of fructose 1,6-diphosphate or fructose 1-phosphate to dihydroxy-

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acetone phosphate and glyceraldehyde 3-phosphate or glyceraldehyde, respectively.

On addition of aldolase to a solution of TNM in buffer or in buffer containing glyceraldehyde 3-phosphate, there is a small increase in absorbance at 350 nm due to oxidation of sulfhydryl groups and nitration of tyrosyl residues of the protein and to non-specific breakdown of TNM, catalyzed by the basic groups of the protein. However, when aldolase is added to a mixture containing the substrate, fructose 1,6-diphosphate, together with TNM, a marked increase in the rate of nitroformate production is observed (Figure 1). A similar increase in nitroformate production is observed in the presence of fructose 1-phosphate and also with dihydroxyacetone phosphate.

A number of control experiments establish the specific dependence of this reaction on the enzymatic aldolase reaction. The marked increase in nitroformate production occurs only when both aldolase *and* substrate are present in the reaction mixture, and only with active enzyme. Moreover, and most important, only substrates for the enzymatic cleavage-condensation reaction enhance nitroformate production. TNM reacts neither with the products of the cleavage reaction, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, nor with aldolase substrates in the presence of proteins other than aldolase. The rate of nitroformate production is proportional to enzyme concentration. The dependence of the rate of nitroformate production on aldolase activity is also apparent from the nonlinearity of the reaction time course (Figure 1) reflecting progressive enzyme inactivation by side chain modification, a process which is also observed in the absence of substrate[13].

The relationship between the rate of nitroformate production and substrate concentration (Figure 2) suggests further that the reaction depends on the formation of an enzyme-substrate complex. This is demonstrated by double-reciprocal plots of the velocity of nitroformate production vs. substrate concentration which are linear for both substrates (Figure 3). The concentration of fructose 1,6-diphosphate or fructose 1-phosphate, resulting in half the maximal rate of nitroformate production, expressed as Km', correspond closely with the value of K_m, as determined by the usual enzymatic cleavage reaction. Essentially the same results are obtained when dihydroxyacetone phosphate serves as the substrate. These results indicate that TNM reacts with an aldolase-substrate complex, specifically with the same complex that leads to the cleavage reaction. Consistent with this view, inorganic phosphate competitively inhibits the TNM reaction with an inhibition constant of the same order of magnitude as that reported for inhibition of fructose 1,6-diphosphate cleavage.

Experiments with limiting amounts of substrate demonstrate that the substrate is consumed by the reaction with TNM in the presence of aldolase, indicating that nitroformate production results from a reaction of TNM with the *substrate* moiety of the enzyme substrate complex.

The location of the TNM-reactive carbanion intermediate in the reaction sequence of aldolase may be deduced from the finding that the reaction of TNM with an aldolase-substrate intermediate occurs with fructose 1,6-diphosphate and fructose 1-phosphate as well as with dihydroxyacetone phosphate. The carbanionic intermediate, therefore, must be located in a segment of the reaction sequence common to all of these substrates. This view is consistent with the reaction mechanism of this enzyme proposed on the basis

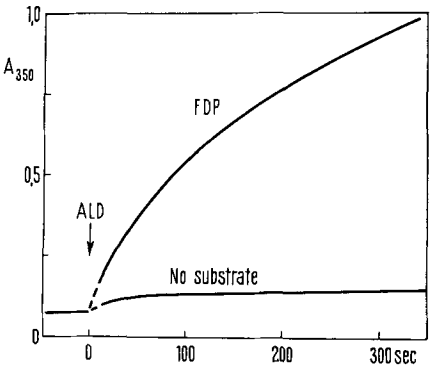

Fig. 1. Production of nitroformate in the presence of aldolase and substrate. Aldolase, 6.95×10^{-4} μmoles (20 μl of a 5.5 mg/ml solution) was added to 1.98 ml of 0.05 *M Tris*-Cl–0.3 *M* NaCl–0.42 m*M* TNM (pH 8.0), 25 °C, in a 1 cm cuvet containing 1 m*M* fructose 1,6-diphosphate (FDP), or no substrate, respectively.

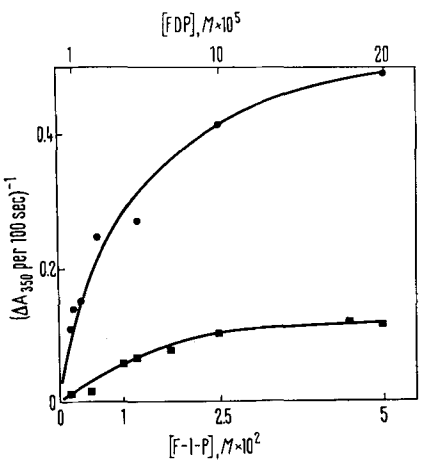

Fig. 2. Rate of nitroformate production by aldolase as a function of substrate concentration. The reaction mixture contained fructose 1,6-diphosphate (●) or fructose 1-phosphate (■) at the indicated concentrations; other conditions were the same as those in Figure 1.

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of different experimental approaches[24]. Thus, in analogy to equation (1) the reaction of TNM with an intermediate aldolase-substrate carbanion can be represented by:

$$\begin{array}{c} \overset{+\,GAP}{\rightleftharpoons} \quad \overset{+H^{\oplus}}{\rightleftharpoons} \\ ALD + FDP \quad ALD\text{–}DHAP^{\ominus} \quad ALD + DHAP \\ \text{(carbanion)} \quad -H^{\oplus} \\ \downarrow + TNM \qquad (3) \\ ALD\text{–}[NO_2 - DHAP] + C(NO_2)_3^{\ominus} \\ \downarrow \\ ALD + [NO_2 - DHAP] \end{array}$$

TNM reacts with the aldolase dihydroxyacetone phosphate carbanion to form a modified enzyme-substrate complex, thereby releasing nitroformate. The enzyme is regenerated by the dissociation of the modified substrate. This derivative, shown in brackets, has been isolated and characterized as an organic phosphate ester differing from dihydroxyacetone phosphate by enzymatic assay and in chromatographic behavior. Equation 3 is not intended to indicate the reaction stoichiometry. Quantitative analysis demonstrates that, under the conditions employed, about 3 moles of nitroformate are produced for every 2 moles of substrate consumed. Interpretation of this finding must await further experimentation, especially the elucidation of the structure of the product.

Chemical probing of the substrate moiety of an enzyme-substrate complex is useful not only in the detection and identification of an intermediate but also to evaluate the effect of varying conditions on the details of the reaction mechanism. Thus, in order to illustrate the possible applications of this approach the TNM-carbanion reaction was employed to examine the effect of carboxypeptidase-treatment on muscle aldolase, to compare the reaction mechanism of two analogous enzymes, i.e. muscle and yeast aldolase, and to elucidate the function of cofactors of yeast aldolase.

2. *Carboxypeptidase-treated muscle aldolase.* Treatment with carboxypeptidase A decreases the fructose 1,6-diphosphate cleavage activity of muscle aldolase to about 5%[25]. Apparently one distinct step, i.e. the formation of the C–H bond of dihydroxyacetone phosphate is affected, its rate being lowered such that it becomes rate-limiting in both fructose 1,6-diphosphate and fructose 1-phosphate cleavage[24].

Applying the TNM-carbanion reaction to carboxypeptidase-treated aldolase the maximal rate of nitroformate production, V', when fructose 1,6-diphosphate serves as the substrate, was found to be 3 times higher than that of native aldolase, while the cleavage reaction is only 5% of that of the native enzyme (Table I). When fructose-1-phosphate is the substrate the rate of nitroformate production is twice that of the native enzyme while the rate of the cleavage reaction remains unchanged. This rate increase of nitroformate production apparently reflects an enhanced concentration of the TNM-reactive intermediate in the steady-state of hexose phosphate cleavage. Thus, these findings are consistent with the carbanion of the dihydroxyacetone phosphate-aldolase complex being the TNM-reactive intermediate and the location of the carboxypeptidase effect to the C–H bond-making step. A decrease in rate of this step will indeed result in an accumulation of the carbanion intermediate (cf. equation 3).

3. *Comparison of carbanion intermediates of yeast and muscle aldolase.* Two classes of enzymes which catalyze the aldol cleavage-condensation reactions of fructose 1,6-diphosphate but which differ in several distinctive features, appear to have evolved independently[26,27]. Class I aldolases, such as muscle aldolase, occur in animals, plants, protozoans and green algae, and, by definition, form Schiff base complexes with substrate

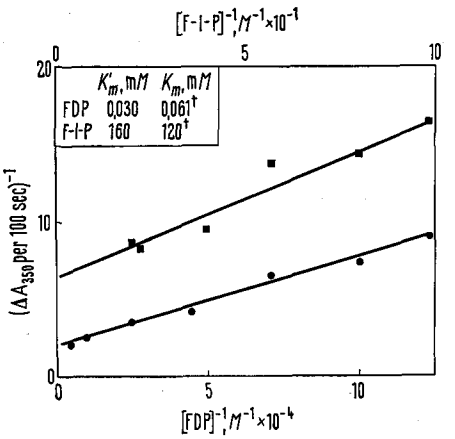

Fig. 3. Double-reciprocal plots of rate of nitroformate production vs. concentration of substrates. Data are from Figure 2, fructose 1,6-diphosphate (●), fructose 1-phosphate (■); K_m' = Michaelis constant with respect to nitroformate production. † from RUTTER et al.[23].

Table I. TNM-Carbanion reaction of native and carboxypeptidase-treated muscle aldolase[a]

Substrate	Native aldolase min⁻¹		Carboxypeptidase-treated aldolase, min⁻¹	
	V'[b]	V[b]	V'	V
Fructose 1,6-diphosphate	60	1900	200	91
Fructose 1-phosphate	19	76	43	95

[a] Prepared as reported previously[19]. [b] V' and V are the maximal molecular activities with respect of nitroformate production and hexose phosphate cleavage, respectively. Conditions were those of Figure 2.

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through an active-site lysyl residue[28]. Class II aldolases, such as yeast aldolase, are found in bacteria, fungi, and blue-green algae and, by definition, require a divalent metal ion for activity[29,30]. Schiff base complexes have not been observed in Class II enzymes. Since yeast aldolase, a Class II enzyme containing zinc, also catalyzes tritium exchange between dihydroxyacetone phosphate and water[31], its analogy to muscle aldolase might extend beyond substrate and reaction specificities to the mechanism of action, i.e. both might operate via intermediary carbanions. The TNM-carbanion reaction has been applied to examine this possibility[20].

Addition of yeast aldolase to a mixture of fructose 1,6-diphosphate and TNM instantaneously initiates the appearance of yellow color due to nitroformate production quite similar to muscle aldolase (cf. Figure 1). Examination along the lines discussed above for the muscle enzyme confirms that the reaction is catalysis-induced. Again, it is specific for aldolase substrates, proportional to enzyme concentration, requires active enzyme, and follows apparent Michaelis-Menten kinetics, K_m' being close to K_m.

In functional terms, as judged by the TNM-carbanion reaction, yeast and muscle aldolase appear to be very similar. With both enzymes, the rate-limiting step for fructose 1-phosphate cleavage seems to occur prior to the carbanion intermediate, fructose 1-phosphate inducing virtually no nitroformate production with the yeast enzyme. The ratio of the rate of nitroformate production, V', to the rate of fructose 1,6-diphosphate cleavage, V, indicates the fraction of the intermediary carbanion occurring in the steady state of the cleavage reaction that is trapped by TNM. Since the experiments with both muscle and yeast aldolase employed the same concentrations of TNM, all these values, V', V, and V'/V, can be compared directly, including the rate of nitroformate production observed in the presence of dihydroxyacetone phosphate which represents an equilibrium rather than a steady-state concentration of the reactive intermediate (Table II). The similarity of all these values suggests that the mechanisms of action of the two enzymes proceed through analogous carbanion intermediates present in both cases in about the same amount and exhibiting similar reactivities toward TNM.

The apparent similarity in reactivity of the enzyme-substrate intermediates of the two aldolases illustrates the decisive effect of the protein environment on the properties of catalytic groups. In the muscle enzyme a Schiff base between the ε-amino group of a lysyl residue and the substrate is formed, and the dihydroxyacetone phosphate carbanion is thought to be resonance stabilized[27]. Yeast aldolase apparently does not form a Schiff base. It has been suggested that the metal ion instead acts as an electrophile inducing carbanion formation, thus serving a function analogous to that of a lysyl residue[26]. The fact that a lysyl-Schiff base and a metal ion complex exhibit the same reactivity is unlikely to be coincidental but may reflect the precise adjustments toward optimal function that these enzyme proteins have undergone during convergent biological evolution. There are a number of reactions known that are catalyzed by a metalloenzyme in one species and by a nonmetalloenzyme in another[32] and detailed comparison of their reaction mechanisms might be a worthwhile endeavor.

4. *Effects of cofactors on yeast aldolase.* As demonstrated with carboxypeptidase-treated aldolase the TNM-carbanion reaction provides a new tool to localize the effect of various cofactors in the reaction sequence of an enzyme. If removal of the cofactor increases the rate of nitroformate production, the cofactor must promote a step occurring after the formation of the carbanion. Conversely, if nitroformate production decreases, carbanion formation must be impaired.

Activation by monovalent cations is another distinctive property of Class II aldolases[26]. The activating effects of potassium ions on cleavage activity and on nitroformate production were found to be closely parallel (Figure 4), suggesting that potassium ions promote an increase in the rate of carbanion formation rather than an acceleration of the steps between carbanion formation and the liberation of dihydroxyacetone phosphate from the enzyme. This being the case, potassium ions should not alter appreciably the rate of hydrogen exchange between dihydroxyacetone phosphate and water. Thus, the 2-fold increase in the rate of tritium-exchange observed in the presence of potassium ions[33] can be contrasted with the more than 10-fold increase in the rate of fructose 1,6-diphosphate cleavage[26] and of nitroformate production (Figure 4).

Table II. TNM-Carbanion reaction with muscle and yeast aldolase

	Muscle aldolase		Yeast aldolase	
	Fructose 1,6-diphosphate	Dihydroxyacetone phosphate	Fructose 1,6-diphosphate	Dihydroxyacetone phosphate
V', min^{-1} [a]	60	71	74	90
V, min^{-1} [a]	1900	–	8800	–
V'/V	0.03	–	0.008	–

[a] V' and V are the maximal molecular activities with respect to nitroformate production and hexose phosphate cleavage, respectively. They were determined for muscle aldolase as in Figures 2 and 3, for yeast aldolase as reported previously[20].

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Consistent with the postulated role of the metal ion as an electrophile inducing carbanion formation, inhibition of the cleavage activity by a chelating agent, EDTA, also completely abolishes nitroformate production. Since the original zinc ions can be replaced by certain ions of the first transition period to result in an active enzyme[30], studies of the relationship between electronegativity of the active site metal ion and reactivity of the carbanion intermediate are an obvious extension of these studies and should provide further insight in the role of this inorganic cofactor.

5. *Related observations, future applications.* Various other observations regarding the specific chemical nature of enzyme-substrate intermediates conform in some respects to the experimental principle outlined above and may serve to emphasize the potential and generality of this approach to enzyme-substrate intermediates.

N-ethylmaleimide has been found to react with a carbanionic enzyme-substrate intermediate of the cystathionine cleavage enzyme of Neurospora[34, 35] and has been used as a carbanion trapping reagent in the reaction of β-chloroglutamic acid with aspartate aminotransferase[36]. The covalent binding of substrates to certain enzymes has been detected by secondary chemical modification of the enzyme-substrate bond. Thus, the Schiff bases of various pyridoxal phosphate dependent enzymes[37], of aldolases, transaldolases[28], and acetoacetate decarboxylase[38] with their respective substrates have been reduced with sodium borohydride to stable secondary amines. Trapping reactions with a nucleophile other than water have been used to examine the acyl and phosphoryl intermediates of hydrolases[39, 40].

These observations had been regarded as rather special cases and the possible general applicability of the approach had not been emphasized. The TNM-carbanion reaction represents an especially useful addition to the already known reactions as it is easily accessible to kinetic analysis. Preliminary results suggest it might be applicable to the examination of the presteady-state of the enzymatic reaction by stopped-flow techniques.

The use of TNM as a reagent for detecting carbanion intermediates apparently is not limited to fructose 1,6-diphosphate aldolases. Reactions with enzyme-substrate complexes of 2-keto-4-hydroxyglutarate-aldolase[41] and of 6-phosphogluconate dehydrogenase have been observed. Other carbon-carbon lyases are likely candidates for study and yet other possibilities are suggested by the results with coenzyme systems, e.g. thiamine and pyridoxal[19].

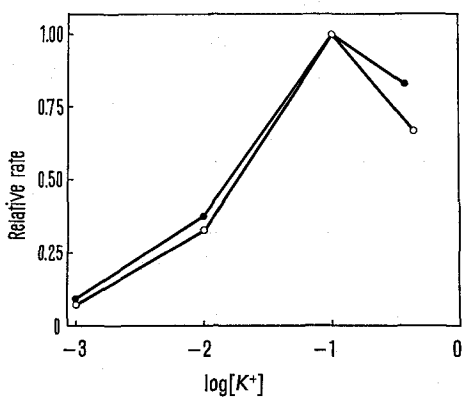
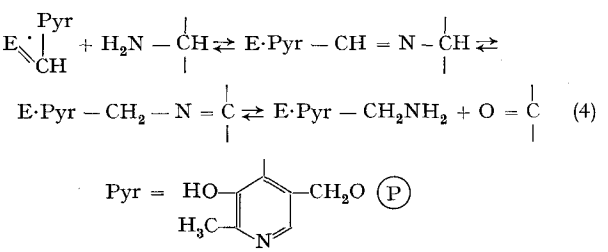


Fig. 4. Effect of potassium on the rates of nitroformate production and fructose 1,6-diphosphate cleavage with yeast aldolase. Rate of nitroformate production (●), and of fructose 1,6-diphosphate cleavage (○)[26]. The reaction mixtures contained potassium chloride at the indicated concentrations.

B. *Chemical probing of the enzyme moiety; catalysis-dependent nitration of aspartate aminotransferase*

According to the concept of multistep catalysis involving a conformationally flexible enzyme[7–10], essential side chain groups of the protein may be expected to change their chemical properties in the course of catalysis. As outlined in the introduction, such catalysis-induced changes in the reactivity of amino acid residues might be detected by probing their response to chemical agents in the presence and absence of substrate. Studies with aspartate aminotransferase have revealed indeed that a functional tyrosyl residue, unreactive in the absence of substrate, undergoes changes in its reactivity in the course of catalysis. In one particular segment of the catalytic pathway it becomes extremely susceptible to chemical modification, and undergoes selective, catalysis-dependent nitration with tetranitromethane[21, 22].

1. *The aminotransferase reaction.* Equation 4 represents the transamination reaction catalyzed by aspartate aminotransferase. The pyridoxal form of the enzyme reacts with an α-amino acid to give an aldimine,



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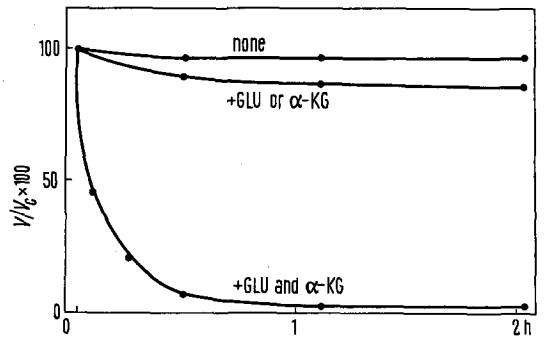

Fig. 5. Catalysis-dependent inactivation of aspartate aminotransferase by TNM. Enzyme, $2.1 \times 10^{-5} M$ in 0.05 *M Tris*-Cl (pH 7.5) was incubated at 22 °C (with $6.0 \times 10^{-4} M$ TNM in the presence of both 70 m*M* L-glutamate *and* 1.75 m*M* α-ketoglutarate, in the presence of either glutamate *or* α-ketoglutarate, and in the absence of any substrate. TNM was added at zero time and at the indicated times transaminase activity was determined using a coupled assay with malate dehydrogenase.

Table III. Aspartate aminotransferase and TNM in the presence and absence of substrates. Amino acid modifications[a]

Substrates	Tyrosyl residues nitrated moles/mole	Sulfhydryl groups oxidized moles/mole	Activity $v/v_c \times 100$
(A) present	1.7	1.7	3
(B) absent	0.7	1.4	95
Δ(A–B)	1.0	0.3	92

[a] Conditions are those of Figure 5. After 1 h the reaction was stopped by addition of 2-mercaptoethanol (0.125 *M*). After dialysis nitrotyrosyl content was determined spectrophotometrically. Amino acid analysis of protein hydrolyzates confirmed that the absorbance at 430 nm originated from 3-nitrotyrosine. Sulfhydryl groups were measured using *p*-mercuribenzoate[43]. The difference between native and modified enzyme is reported.

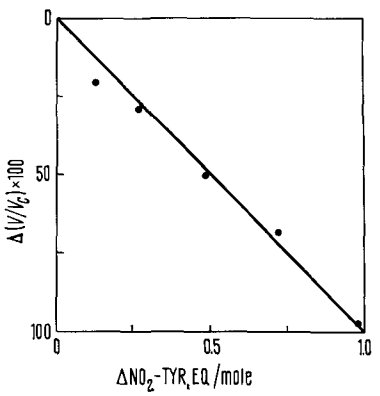

Fig. 6. Aspartate aminotransferase and TNM: Catalysis-dependent inactivation and tyrosyl nitration. Conditions are those of Figure 5. After different times the reactions were stopped by addition of 2-mercaptoethanol. Activity and nitrotyrosyl content were determined as in Table III. The difference in activity of enzyme incubated with TNM plus the substrate pair and enzyme incubated with TNM alone is plotted vs. the difference in degree of nitration of the same samples.

which tautomerizes to a ketimine. Subsequent hydrolysis yields the pyridoxamine form of the enzyme and the corresponding α-keto acid. Reversal of the reaction with another α-keto acid completes one cycle of transamination[42]. If a pair of structurally analogous substrates, such as glutamate and α-ketoglutarate are employed, transamination occurs without any net change in the concentration of either substrate and an equilibrium concentration of each intermediate will exist in the reaction mixture. This situation allows examination of catalysis-induced reactivity of enzyme side chains.

2. *Reaction with TNM in the presence and absence of substrates.* When aspartate aminotransferase is incubated with TNM in the absence or presence of substrates, the effect on enzymatic activity is quite different (Figure 5). In the absence of substrates neither the pyridoxal nor the pyridoxamine form of the enzyme is inactivated appreciably. In the presence of either glutamate or α-ketoglutarate the enzyme is inactivated very slowly. In contrast, in the presence of the substrate pair, glutamate *and* α-ketoglutarate, the enzyme is rapidly inactivated apparently reflecting the modification of an essential group which becomes highly reactive toward TNM in the course of the enzymatic reaction.

3. *Nature of the modified essential group.* The inactivation can be ascribed to the catalysis-dependent nitration of a particular tyrosyl residue (Table III). In the presence and absence of substrates both tyrosyl and sulfhydryl residues have been found to be modified consistent with the reported specificity of TNM[12,13]. However, in the presence of substrates one additional tyrosyl residue is nitrated. Further, nitration of this tyrosyl residue correlates linearly with the loss of activity over the entire range of catalysis-induced inactivation (Figure 6). Hence, this tyrosyl residue would seem to be the essential group exhibiting catalysis-induced reactivity. Gross structural alterations in the modified protein have been ruled out by sedimentation analysis and by circular dichroic spectra.

4. *The coenzyme after catalysis-dependent inactivation.* Coenzyme alteration is not the cause of inactivation. The absorption spectrum of inactive aminotransferase, nitrated in the presence of substrates, exhibits a nitrotyrosine absorption maximum at 428 nm and one at 330 nm, due to bound coenzyme (Figure 7). Comparison with the native pyridoxamine form of the enzyme suggests that the coenzyme of the inactive enzyme exists entirely in the pyridoxamine form. The spectra of both the pyridoxal and the pyridoxamine enzymes (not shown in Figure 7), incubated with TNM in the absence of substrates, are unchanged in the

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region of coenzyme absorption compared to the native enzymes. The cofactor bound to the inactivated enzyme has been isolated and its absorption characteristics, chromatographic behavior and ability to reactivate native apoenzyme have confirmed its identity with pyridoxamine phosphate.

The circular dichroic spectrum of the inactivated enzyme also suggests that neither the coenzyme nor its asymmetric mode of binding have been affected by the modification (Figure 8). The inactivated enzyme has retained the positive Cotton effect at 333 nm typical of native pyridoxamine aminotransferase.

Thus, the coenzyme of the inactivated enzyme appears indeed to be irreversibly trapped in its pyridoxamine form. No evidence for conversion of the inactivated enzyme to the pyridoxal form or to an aldimine intermediate was obtained even after exhaustive dialysis against an excess of α-ketoglutarate. Thus, it would appear that the inactivation reaction occurs during or after the transition of aldimine to ketimine.

5. *Catalysis-linked reactivity changes of the essential tyrosyl residue.* The reactivity of the essential tyrosyl residue in the various intermediates of the catalytic reaction (cf. equation 4, which displays only the most important intermediates) has been studied using single substrates, competitive inhibitors and substrate analogs.

The pyridoxamine enzyme in the presence of glutamate or the pyridoxal enzyme in the presence of α-ketoglutarate are inactivated very slowly by TNM (Table IV). Binding of competitive inhibitors[44] also fails to induce rapid inactivation with both the pyridoxal and the pyridoxamine form of the enzyme. Thus, by itself the formation of adsorption (Michaelis) complexes (not shown in equation 4) seems insufficient to induce reactivity toward TNM leaving the aldimine or ketimine as the most probable TNM-reactive intermediate. The substrate analog, α-methylaspartate, forms an aldimine complex with the pyridoxal enzyme. However, since it lacks an α-hydrogen, the aldimine complex cannot tautomerize to the ketimine[45]. The presence of α-methylaspartate induces some enhancement in the rate of inactivation but this rate is still 15 times slower than in the presence of the substrate pair when both aldimines and ketimines are formed (Figure 9). Thus, rapid inactivation seems to occur only when the enzyme-substrate complex shuttles through the total catalytic reaction sequence.

Table IV. Inactivation of aspartate aminotransferase by TNM in the presence of substrates and competitive inhibitors[a]

Addition	Activity after 1 h, $v/v_c \times 100$	
	Pyridoxal enzyme	Pyridoxamine enzyme
Glutamate	83[b]	73
α-Ketoglutarate	88	72
Maleate	91	–
Glutarate	89	92
Phthalate	89	–
None	96	81

[a] Conditions were as in Figure 5. Concentrations of maleate, glutarate, and phthalate are 45, 60, and 100 mM, respectively. [b] At low enzyme concentration ($4 \times 10^{-7} M$).

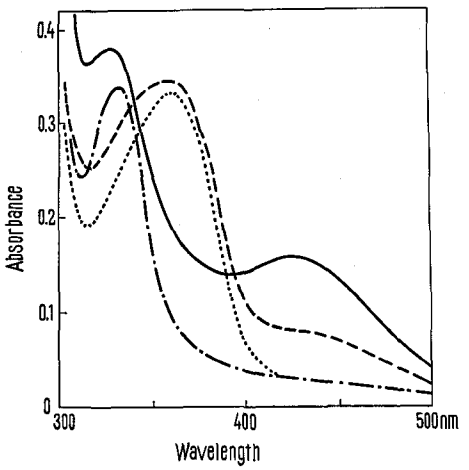

Fig. 7. Absorption spectra of aspartate aminotransferase nitrated in the presence and absence of substrates and of native pyridoxamine and pyridoxal enzyme. Conditions for nitration were those of Table III. The enzyme concentration is $1.8 \times 10^{-5} M$ in 0.05 M *Tris*-Cl (pH 8.5); incubated with TNM in the presence of glutamate and α-ketoglutarate (——), residual activity 3% of control, 1.7 moles of nitrotyrosine per mole; pyridoxal enzyme incubated with TNM in the absence of substrates (– – –), residual activity 93% of control, 0.7 moles of nitrotyrosine per mole; native pyridoxamine enzyme (– · –): native pyridoxal enzyme (. . . . .).

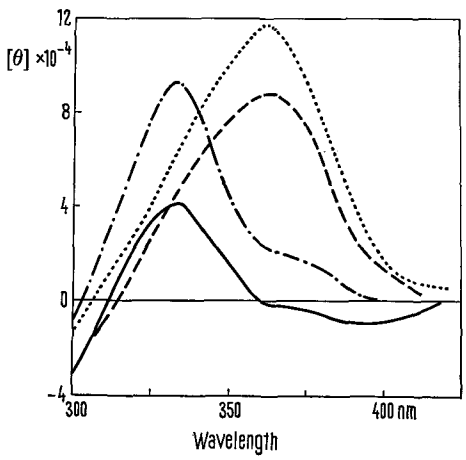

Fig. 8. Circular dichroic spectra of aspartate aminotransferase nitrated in the presence and absence of substrates and of native pyridoxamine and pyridoxal enzyme. Conditions and notations are the same as in Figure 7: nitrated in the presence of the substrate pair (——) (the origin of the decrease in amplitude and of the newly generated Cotton effect near 395 nm is unclear and remains to be elucidated); pyridoxal enzyme nitrated in the absence of substrates (– – –); native pyridoxamine enzyme (– · –); native pyridoxal enzyme (. . . .). Enzyme concentrations were $2 \times 10^{-5} M$ in 0.05 M *Tris*-Cl (pH 8.5) in a 1 cm cell.

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Apparently, the reactivity of the functional tyrosyl residue of aspartate aminotransferase is altered synchronously with catalysis. The loss of enzymatic activity accompanying tyrosyl nitration suggests that such syncatalytic reactivity changes are integral features of the mechanism of action of aspartate aminotransferase.

In order to assess the reactivity of the various intermediates, the rates of reaction with TNM are compared with that of tyrosyl model compounds (N-acetyltyrosine, N-acetyltyrosine ethyl ester, and N-acetyltyrosine amide) (Table V). Since inactivation of the enzyme correlates with nitration of one tyrosyl residue (Figure 6), the rate of inactivation represents the rate of nitration of the critical tyrosyl residue. Inactivation of the enzyme in the absence of any substrate is markedly slower than reaction of the tyrosyl model compounds suggesting that the protein environment precludes nitration of the critical residue. In the presence of α-methylaspartate, when aldimine intermediates are formed, the essential tyrosyl residue apparently becomes available to TNM and is nitrated at a rate comparable to that of the model. However, in the presence of the substrate pair, when ketimine intermediates are formed, the rate of nitration is increased 15 times over that of a normal tyrosine. Apparently, under these conditions the tyrosyl residue is activated.

The rates of enzyme inactivation and of the reaction of TNM with tyrosine model compounds were determined as a function of pH, in order to elucidate the mechanism of syncatalytic activation of this essential residue (Figure 10). Under all conditions with or without substrates, or with α-methylaspartate, the rates of inactivation, i.e. of nitration of the essential tyrosyl residue, are largely independent of pH. This contrasts with the rates of reaction between TNM and the tyrosyl model compounds which are markedly pH dependent. Apparently, the reactivity of the functional residue is shielded from pH effects, and in the native enzyme and in all intermediate enzyme-substrate complexes is determined rather by environmental effects originating from neighboring protein side chains and/or the coenzyme.

The changes in reactivity from one catalytic stage to another, therefore, seem to reflect alterations in the protein-coenzyme environment of the functional residue in the course of catalysis. Such environmental changes may result either from conformational changes during the transition from one intermediate to another or from direct involvement of the critical group in bond breaking and making processes without conformational changes of the protein. However, in the present case, the latter mechanism appears unlikely to account for the catalysis-induced abolition of the 'buried', unreactive character of the functional residue that preexists the addition of substrate. Thus, the syncatalytic reactivity changes of this residue seem

Table V. Rates of reaction with TNM[a]

Reactant	Rate constant $k_{app} \times 10$, min^{-1}
Tyrosine model compounds[b]	0.06
Aspartate aminotransferase:	
Native	<0.01[c]
In the presence of α-methylaspartate (aldimine complex)	0.08[c]
In the presence of glutamate and α-ketoglutarate (aldimine and ketimine complexes)	1.2[c]

[a] Concentration of reactant was $2.1 \times 10^{-5} M$, of TNM 0.6 mM in 0.05 M *Tris*-Cl (pH 7.5) at 22 °C. [b] N-acetyltyrosine, N-acetyltyrosine ethyl ester, N-acetyltyrosine amide. The indicated rate is the rate of nitroformate production measured spectrophotometrically, $\varepsilon_{350} = 14.400$[17]. [c] Rate of inactivation.

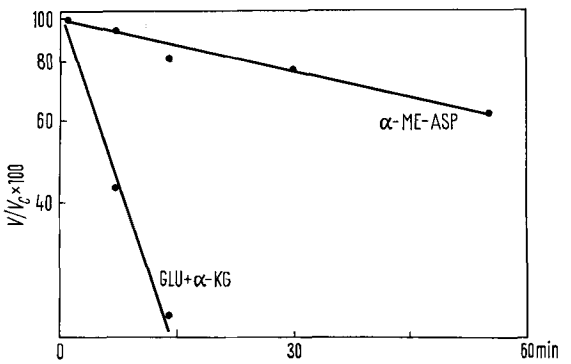

Fig. 9. Inactivation of aspartate aminotransferase with TNM. Comparison of the effect of 0.14 M D,L-α-methylaspartate with that of glutamate plus α-ketoglutarate. Conditions were those of Figure 5. While the rate of inactivation is 15 times slower, loss of activity still correlates with modification of a specific tyrosyl residue.

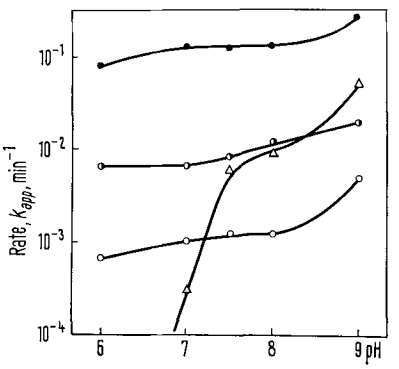

Fig. 10. Inactivation of aspartate aminotransferase by TNM and reaction of tyrosyl model compounds with TNM, pH-dependence. Conditions are those of Table V. At pH 7, 8, 9 the buffer was *Tris*-C and at pH 6 it was *Tris*-acetate. Rate of enzyme inactivation in the presence of glutamate and α-ketoglutarate (●), in the presence of α-methylaspartate (◑), and in the absence of substrate (○); rate of nitroformate production with tyrosyl model compounds (△). Analysis of the enzyme, inactivated at pH 6 in the presence of the substrates, revealed also an extra tyrosyl residue nitrated indicating that over the entire pH range the rate of inactivation in the presence of substrates may be equated with the rate of nitration of the functional tyrosyl residue. No significant differences in rates were observed between N-acetyltyrosine, N-acetyltyrosine ethyl ester, and N-acetyltyrosine amide.

rather to result from conformational alterations of its protein-coenzyme environment.

Syncatalytic activation is also observed with other probing reagents. Qualitatively, the same positive effect of substrates is observed on the rate of inactivation with iodine, though this reaction is complicated by the fact that the enzyme is inactivated also in the absence of the substrates and by apparent initial substrate protection. However, a catalysis-dependent component of the inactivation in the presence of substrates is clearly discernible (Figure 11).

On the basis of other lines of evidence, IVANOV and KARPEISKY[9] have proposed a detailed multi-stage mechanism of action of aspartate aminotransferase that involves a tyrosyl residue participating in topological alterations of the enzyme-coenzyme-substrate system. Their view of multi-stage catalysis emphasizes 'stabilization-orientation-change of conditions by positional change' as its principal features and is consistent with the observation of syncatalytic side chain activation induced by conformational changes. However, the correspondence of the particular tyrosyl residue which is syncatalytically activated to the functional tyrosyl residue postulated by these workers remains to be established.

Further, TURANO and collaborators have found that nitration of a tyrosyl residue in apoaspartate aminotransferase prevents recombination with the coenzyme (C. TURANO, personal communication). Again, additional experimentation is required to elucidate the relation, if any, of this residue with the one that is syncatalytically activated.

The catalysis-dependent nitration observed with aspartate aminotransferase suggests that it might be possible to distinguish three different modes of side chain activation, all three perhaps related to catalytic function.

First, unusual reactivity of certain groups of the native enzyme in the absence of substrates has been observed in a large number of enzymes[6]. Such 'atypical' chemical behavior is apparently characteristic of biologically active proteins and seems to be conditioned by the native protein environment.

Second, enhanced reactivity of certain groups has also been observed consequent to substrate or substrate analog binding, possibly reflecting the adaption of the active site topography to effective catalysis[46].

Lastly, syncatalytic side chain activation as observed with aspartate aminotransferase appears as a still more complex phenomenon, since it occurs not on binding of substrate but only in the course of the ensuing stages of catalysis, i.e. during the bond making and breaking processes.

Further examples of syncatalytic side chain activation may not be easily predicted as many factors possibly critical for these reactions are still unknown. However, an apparently related phenomenon has been observed with catalase where a presently unknown but specific residue becomes reactive in the catalase-H_2O_2 complex I and reacts irreversibly with 3-amino-1,2,4-triazole yielding an inactive enzyme[47,48].

Awareness of the phenomenon of syncatalytic side chain activation may lead to a more detailed examination of chemical modifications, carried out in the presence of substrates. As the effect of substrates on the modifying reaction in most cases has been evaluated only by means of alterations of enzymatic activity, differences in the degree of modification of a particular functional amino acid residue might often have remained undetected. Further, the elucidation of the structure of an increasing number of active sites by X-ray crystallography might stimulate the design of such experiments on a rational basis.

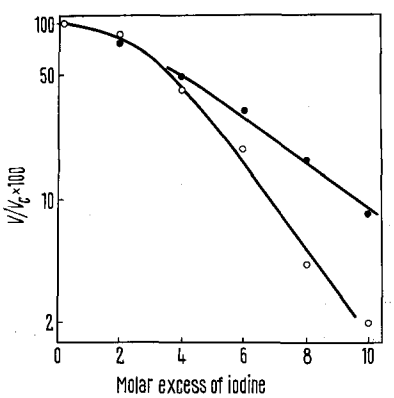

Fig. 11. Inactivation of aspartate aminotransferase by iodine in the presence (○) and absence(●) of substrates. Aliquots of 0.05 *M* iodine in 0.5 *M* potassium iodide were added successively to give the molar excesses indicated. Activity was determined 3 min after each addition of iodine. Other conditions were as in Figure 5.

Conclusion

Chemical probing of enzyme-substrate intermediates when applied to aldolases and aspartate aminotransferase, has proven to be a valuable experimental approach to the dynamic features of enzyme catalysis. The studies demonstrate that enzymatic reactions may proceed through intermediates which distinguish themselves by their specific chemical properties on both the enzyme and substrate moiety of the enzyme-substrate complex. Chemical probing with a suitable reagent can detect such intermediates and render them accessible to examination of their contribution to the enzymatic reaction.

In the reaction mechanisms of muscle aldolase (a class I aldolase) and yeast aldolase (a class II aldolase) very similar carbanion intermediates have been detected. Apparently, the different structures of these

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[48] B. B. L. AGRAWAL and E. MARGOLIASH, Fedn. Proc. *28*, 405 (1969).

enzymes have been adapted to a closely analogous functional behavior. Intermediates with critically tuned chemical properties may be essential for the efficiency of enzyme catalysis, and this close functional similarity might be the result of an intense selection pressure exerted on these two proteins during biological evolution.

The results with aspartate aminotransferase provide direct experimental indication of a possible mechanism for a multistep enzyme catalysis. The syncatalytic activation of a particular functional residue, produced by catalysis-linked conformational alterations of the enzyme-coenzyme-substrate complex, might well reflect the generation of a transient active site topochemistry optimally adapted for stabilization of an intermediate and/or catalysis of the neighboring steps in a multistep enzymatic reaction. Thus, the triad: conformational adaptability, syncatalytic changes in reactivity of functional groups, multistage reaction, might be interdependent features of enzyme catalysis[49, 50].

Zusammenfassung

Die Untersuchung von Enzymsubstratkomplexen auf katalyse-induzierte Änderungen der chemischen Reaktivität hat sich als ein aufschlussreicher experimenteller Zugang zum Mechanismus enzymatischer Reaktionen erwiesen. Ternäre Systeme bestehend aus Enzym, Substrat und einem geeigneten Reagens (Tetranitromethan = TNM) sind auf katalyse-abhängige Reaktionen geprüft worden, die nur im vollständigen System, jedoch nicht mit Reagens und Enzym allein oder Reagens und Substrat allein beobachtet werden. Untersuchungen mit Aldolasen und Aspartat-Aminotransferase haben ergeben, dass katalyse-induzierte Reaktivität sowohl auf dem Substrat- als auch auf dem Enzymteil eines Enzym-Substratkomplexes nachgewiesen werden kann.

Im Reaktionsmechanismus von Muskelaldolase (eine Lysinaldolase) und von Hefealdolase (eine Metallaldolase) lässt sich mit TNM ein intermediäres Carbanion des Substrats nachweisen. Die TNM-carbanion-Reaktion lässt sich spektralphotometrisch verfolgen und ist benutzt worden, um carboxypeptidase-behandelte Muskelaldolase und den Effekt von Cofaktoren auf die Hefealdolase zu untersuchen.

Katalyse-induzierte Reaktivitätsveränderungen einer Enzymseitenkette sind bei der Aspartat-Aminotransferase beobachtet worden. Ein essentieller Tyrosylrest, der in der Abwesenheit von Substrat nicht mit TNM reagiert, wird ungewöhnlich reaktiv im Laufe der Katalyse und ermöglicht dabei seine selektive Nitrierung.

Die katalyse-synchrone oder synkatalytische Aktivierung dieses Aminosäurerests scheint ein integraler Bestandteil des katalytischen Mechanismus von Aspartat-Aminotransferase zu sein und wird vermutlich durch katalyse-induzierte Konformationsänderungen des Enzym-Coenzym-Substratkomplexes hervorgebracht. Mögliche funktionelle Zusammenhänge synkatalytischer Reaktivitätsänderungen funktioneller Gruppen mit der Konformationsflexibilität des Enzymproteins und dem Vorkommen metastabiler Zwischenprodukte in der Enzymkatalyse werden diskutiert.

[49] Acknowledgements. I am grateful to J. F. Riordan for his collaboration throughout this work. I would also like to thank Prof. B. L. Vallee for his advice and encouragement and J. H. Kägi for helpful discussions. The excellent technical assistance of Charlotte Hart is gratefully acknowledged.

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SPECIALIA

Bazzanenol, a New Sesquiterpene Alcohol Having a Skeleton of Bicyclo[5.3.1] Undecane System from Hepaticae, *Bazzania pompeana* (Lac.) Mitt.[1]

In a previous paper[2], it was reported that a sesquiterpene hydrocarbon of a new carbon skeleton, bazzanene, was isolated from an essential oil of *Bazzania pompeana* (Lac.) Mitt. (Japanese name Ômukadekoke) belonged to Lepidoziaceae, and its structure was determined as formula (I).

From the same essential oil, subsequently, a new sesquiterpene alcohol was isolated and its structure was determined as 2, 6, 6-trimethyl-8-methylene bicyclo[5.3.1]-undec-2-en-4-ol (II). For this alcohol we propose the name bazzanenol, and here present the evidence for the structural assignment.