EVIDENCE FOR THE PRESENCE OF A DISTINCT SUBSITE FOR BINDING THE DISTAL CARBOXYL GROUP OF DICARBOXYLATE SUBSTRATES AND ITS ROLE IN THE CATALYTIC ACTIVITY OF ASPARTATE AMINOTRANSFERASE[1].

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Received March 20, 1972

Summary: Rates both of the α,β-elimination of β-chloro-L-alanine and the transamination of L-alanine which are catalyzed by the pyridoxal form of the supernatant isozyme of aspartate aminotransferase were greatly accelerated by the presence of formate. The rate of inactivation of the enzyme occurring during the α,β-elimination reaction was also strikingly enhanced by high concentration of formate. A unified explanation for the formate-mediated rate-enhancement of these catalytic processes was attempted by assuming the presence of a discrete subsite within the enzyme active region which binds formate anion and would normally bind the distal carboxyl group of the natural substrate(L-glutamate or L-aspartate). Blocking of this subsite by a carboxylate may induce a conformational change of the catalytic site which favors further chemical reactions in the catalytic process. Thus the present observations provide additional evidence for the active participation of the apparently non-reacting region of a substrate in enzymic catalysis.

INTRODUCTION

Participation of the apparently nonreacting region of a substrate in the catalytic process was well documented in the finding that the rate of trypsin-catalyzed hydrolysis of acetylglycine ethyl ester was enhanced nine folds by the presence of ethylammonium ion (1). An analogous evidence was presented by Saier and Jenkins (2) who observed a 1.2-fold stimulation of alanine aminotransferase-catalyzed transamination and 7-fold stimulation of aspartate aminotransferase-catalyzed alanine transamination by the presence of formate.

During the course of a study on the aspartate aminotransferase-catalyzed α,β-elimination reaction (3), the rate of α,β-elimination of β-chloro-L-alanine has been found to be accelerated by formate or acetate ions, particularly with the supernatant isozyme.

The present communication describes the striking rate enhancement by

1. L-Aspartate: 2-oxoglutarate aminotransferase [EC. 2.6.1.1]

formate ion of (I) α,β-elimination of β-chloro-L-alanine, (II) inactivation of the enzyme occurring during the enzymic catalysis and (III) transamination with L-alanine.

EXPERIMENTAL PROCEDURES

[Materials] The supernatant and mitochondrial isozymes of aspartate aminotransferase were isolated from pig heart muscle by a modification of the procedure described for beef liver enzymes (4). The α form of the supernatant isozyme was obtained as described (5). Lactate dehydrogenase was purified from pig heart muscle as described (6). β-Chloro-L-alanine was synthesized according to the procedure described by Fischer and Raske (7).

[Assay for α,β-elimination of β-chloro-L-alanine] Aspartate aminotransferase catalyzes the equimolar formation of pyruvate, ammonia and chloride from β-chloro-L-alanine (8). The activity of this reaction was, therefore, determined by measuring the rate of decrease in absorbance at 340 nm of NADH in a coupled system containing lactate dehydrogenase. Spectrophotometric measurements were performed in a Hitachi spectrophotometer model 124 with a recorder.

[Determination of inactivation rate] The rate of pyruvate formation in the same assay medium as described above decreased with time. Since this decrease in the rate of pyruvate formation was found to result from an irreversible inactivation of the enzyme during the α,β-elimination reaction[2], the slope of the progress curve should represent the remaining enzyme activity. Thus the pseudo-first order rate constant, k, was obtained from the plot of log (slope) against time, using the equation $k=\frac{0.693}{t_{1/2}}$ where $t_1/2$ is the time that is required for inactivating a half of the enzyme present.

RESULTS AND DISCUSSION

At the earlier stage of this investigation, Tris hydrochloride buffer was employed in the reaction medium. When Tris hydrochloride was replaced by Tris acetate, the rate of α,β-elimination reaction as well as the concomitant inactivation of the enzyme was found to be greatly accelerated. Therefore, a possi-

2. Details of analyses on the inactivation process and the inactivation product will appear elsewhere. The preliminary result was presented (8).

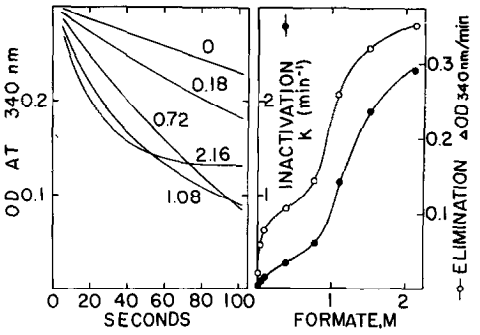

Fig. 1. Effect of formate on rates of α,β-elimination and concomitant inactivation. Reaction mixtures contained, in a total volume of 1.5 ml, 13.3 mM β-chloro-L-alanine, potassium formate as indicated, 23 μg of the supernatant aspartate aminotransferase, 0.06 mM NADH, 2 μg (ca. 1 unit) lactate dehydrogenase and 0.1 M potassium phosphate buffer, pH 7.5. Reaction was initiated by adding the aminotransferase and followed at 20°. Dilution of the formate concentration resulting from the addition of other reactants was only 1.07-fold and data were not corrected for this dilution.
Left : time course of pyruvate formation from β-chloro-L-alanine.
Right : variation of rates of α,β-elimination and inactivation with formate concentrations.

ble acceleration of the rate of these reactions by monocarboxylate anions was examined. Among formate, acetate and propionate, formate was most effective. Fig. 1 illustrates the effect of formate on the rate of α,β-elimination and that of the inactivation of the enzyme. The presence of 2 M formate increased the rate of α,β-elimination of β-chloro-L-alanine some twenty times, and more strikingly, the rate of the inactivation of the enzyme several hundredfolds The irreversible inactivation of the enzyme results from the covalent incorporation of the three carbon moiety from β-chloro-L-alanine (8). That formate binds to the substrate binding site of the enzyme was clearly shown by Jenkins and D'Ari (9). In fact, little activity of normal transamination between α-ketoglutarate and L-aspartate was observed in the presence of 2 M formate (see Table I). The Michaelis constant for β-chloro-L-alanine did not seem to be appreciably affected by the presence of formate[3]. The sigmoidal nature of the saturation curve remains to be explained.

3. The Michaelis constant for β-chloro-L-alanine in the α,β-elimination reaction is very large (~0.5 M). In the presence of high concentration of formate, the enzyme is very rapidly inactivated. Technical difficulties arisin from these facts hampered a precise estimation of Michaelis constants.

These findings indicate that the binding of formate ion to the enzyme active center increases the rate of withdrawal of α-proton from the bound β-chloro-L-alanine (a postulated rate determining step in the aspartate aminotransferase-catalyzed α,β-elimination reaction (10)) and also increases even further the efficiency of nucleophilic attack to the β-carbon atom of the bound substrate by some nucleophilic side chain of an amino acid residue in the catalytic site.

A common intermediary step in catalytic transamination and α,β-elimination reactions is the abstraction of α-proton of the substrate (11). Hence, it was of interest to test the action of formate ion in transamination reaction. For

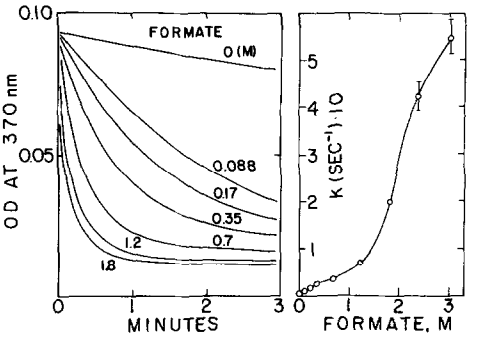

Fig. 2. Formate-induced acceleration of the rate of conversion of the pyridoxal form of the supernatant aminotransferase to the pyridoxamine form by L-alanine. Reaction mixtures contained, in a total volume of 1.2 ml, 1 mg of the supernatant aspartate aminotransferase in pyridoxal form, potassium formate as indicated, and 0.1 M potassium phosphate buffer, pH 7.5. Reaction was initiated by adding 0.05 ml of 1.0 M L-alanine and absorbance change at 370 nm was followed at 20°.
Left : time course of decrease in the pyridoxal form enzyme.
Right : variation of the rate of transamination with formate concentrations.

this purpose, the effect of added formate was examined on the rate of conversion of the pyridoxal form of the enzyme to the pyridoxamine form by L-alanine. As can be seen from Fig. 2, a striking stimulation in the rate of transamination with L-alanine was observed in the presence of formate. Dependency of its rate on the formate concentration was similar to that observed previously in the α, β-elimination reaction and the inactivation process.

Table I summarizes the results obtained in the present investigation. In the experiment described in Fig. 1 and Fig. 2, L-alanine and β-chloro-L-alanine were used at suboptimal concentrations. When extrapolated to an infinite concentration of these substrates, maximal values of k_{CAT} would be of an order of

TABLE I. EFFECT OF FORMATE ON ASPARTATE AMINOTRANSFERASE-CATALYZED REACTIONS.

The rates of α,β-elimination and concomitant inactivation were determined under the conditions described for Fig.1. The rate of transamination with L-alanine was obtained as described for Fig.2. The activity of the normal transamination was determined by measuring the absorbance change at 280 nm of the reaction mixture containing,in 1.5 ml,20 mM L-aspartate,2 mM α-ketoglutarate, 1 μg of the supernatant enzyme and 50 mM Tris hydrochloride buffer(or 3 M potassium formate), pH 8.0.

TYPE OF REACTION	$HCOO^-$	k_{CAT}*1		
		S *2		M
α,β-ELIMINATION	0 M	0.17	(SEC^{-1})	0.50
	0.4	1.0		0.46
	2.0	3.2		0.10
INACTIVATION	0	0.006	(MIN^{-1})	0.06
	0.4	0.28		0.33
	2.0	2.34		-
ALANINE TRANSAMINATION	0	0.002	(SEC^{-1})	0.0009
	0.35	0.03		-
	3.0	0.55		0.024
NORMAL TRANSAMINATION	0	95	(SEC^{-1})	-
	3.0	2		-

*1 k_{CAT} was expressed as moles of products formed per mole of monomeric unit of the enzyme per second or minute.
*2 S:supernatant enzyme, M:mitochondrial enzyme.

10 sec^{-1} for the transamination and of an order of 100 sec^{-1} for the α,β-elimination reaction at 20°. Thus the presence of 3 M formate raised the rates of these reactions up to a level comparable to that ($\sim$100 sec^{-1} at 20°) of trans amination with a normal substrate, L-aspartate or L-glutamate. The finding that both of the α,β-elimination reaction with β-chloro-L-alanine and the transamination reaction with L-alanine were accelerrated by formate anion with similar concentration dependency (see Fig. 1 & 2) strongly supports the idea that formate acts on an intermediary step common to these two types of reactions, presumably the abstraction of α-proton of the enzyme-bound substrates. In order to explain this effect, we assume the presence of a formate-binding subsite within the active region of the enzyme. Combination of formate anion with this site induces a conformational change of the active region to result in bringing a base (X in Scheme I) derived from the enzyme protein in juxtaposition to the α-hydrogen of the enzyme-bound substrate or in enhancing the basicity of this proton-withdrawing side chain of an amino acid residue in an undefined fashion.

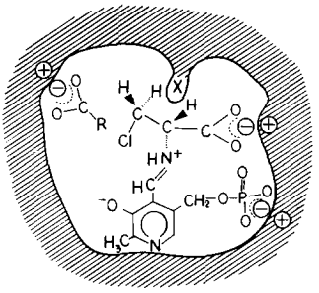

Fig. 3. Schematic representation of the active site of aspartate aminotransferase. Monocarboxylate anion (R : H or CH_3) is assumed to bind via ionic interaction with some cationic group (⊕) within the active site. X is a postulated base or nucleophile which is supplied by an amino acid side chain in the active site.

A striking acceleration of the rate of transamination with L-alanine by the presence of formate leads us to speculate that the formate binding site might be a discrete subsite which normally binds the β-(or γ-) carboxyl group of L-aspartate (or L-glutamate) and blocking of this site by the carboxyl group induces a favorable conformational change to the catalytic site to facilitate further chemical reactions in the catalytic process as discussed above. Thus the present observation may constitute a molecular basis for the apparently strict substrate specificity of aspartate aminotransferase.

Although the affinity of this proposed subsite for formate ion is apparently quite low as judged from its saturation curves for reactions with β-chloro-L-alanine and L-alanine, the affinity for β(or γ)-carboxyl group within the molecule of a normal substrate would be reasonably high since the subsite is expected to be structurally well fit to the built-in carboxylate of normal substrates and binding of the built-in carboxylate is greatly facilitated by an increase in its local concentration which results from the anchoring of the substrate molecule through other linkages, i.e., aldimine linkage to the bound coenzyme and presumably, ionic association of α-carboxyl group with some cationic grouping in the active site.

Concerning the formate-acceleration of the inactivation of the enzyme which occurs during α,β-elimination of β-chloro-L-alanine, we assume that in the inactivation process the α-proton-withdrawing base acts also as a nucleophile which attacks the β-carbon atom of the bound β-chloro-L-alanine and this

nucleophilic attack is accelerated by an increase in the efficiency (or the turnover) of the base which is induced upon combination of formate ion to the postulated subsite.

It is of interest to note that with the mitochondrial enzyme the presence of formate increased only the inactivation rate but did not exert appreciable effect on the rate of the α,β-elimination reaction and, in addition, the rate-acceleration of alanine transamination by formate was much less compared with the supernatant isozyme (Table I). These findings might indicate that the active site of the mitochondrial enzyme is structurally less capable of accomodating formate ions in such a way as to give favorable effects on the catalytic process. This difference in the effect of formate between these isozymes may perhaps be related to the fact that the mitochondrial isozyme prefers 4-carbon dicarboxylates as its substrates whereas the supernatant isozyme 5-carbon dicarboxylates (4, 12).

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