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NEUROSPORA CRASSA INVERTASE

A STUDY OF AMINO ACIDS AT THE ACTIVE CENTER

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Summary

1. The effects on *Neurospora crassa* invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) of a variety of group specific reagents and other potential inhibitors were determined during a search for an irreversible inhibitor of the enzyme. Aniline, pyridoxal, enzyme substrate and products did not inactivate invertase under reducing conditions. Bromoacetic acid, iodoacetic acid, iodoacetamide, *p*-chloromercuribenzoate, hydroxylamine and 2-hydroxy-5-nitrobenzyl bromide were also ineffective. Iodine was the only reagent which irreversibly inhibited invertase.

2. Invertase was rapidly inactivated by low concentrations of iodine, indicating specific inhibition. However, the enzyme could not be protected from this inactivation by substrate. It was not reactivated by mercaptoethanol or cysteine.

3. Experiments on the uptake of radioactive iodine demonstrated that invertase is not iodinated under the conditions of iodine inactivation.

4. The sedimentation ($S_{20,w}$) value of invertase was not altered by iodine inactivation. One-dimensional electrophoresis and finger-printing of tryptic digests revealed no differences between iodine treated and untreated invertase. There was no loss of carbohydrate from this glycoprotein during iodine inactivation.

5. Standard amino acid analyses of iodine-inactivated invertase showed some loss of tyrosine and a trace amount of methionine sulfone. Attempts to demonstrate oxidation of methionine to the sulfone, through modification of the procedure for preparation of samples for analysis, were unsuccessful. However, oxidation of half-cystine was indicated and further loss of tyrosine noted.

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A hypothesis is advanced that half-cystine is oxidized by iodine to a normally unstable oxidation state which is maintained and protected by its protein environment and that loss of tyrosine may be an artifact caused by the presence of this residue during acid hydrolysis.

Introduction

Neurospora crassa invertase, an intramural enzyme, catalyzes the hydrolysis of the β-fructoside bond in sucrose and related molecules [1]. The enzyme is inhibited by the products of catalysis [2], glucose and fructose, by aniline [2,3], by *p*-chloromercuribenzoate [3], by certain metal ions [3] and by pyridoxal [4]. Inhibition is, in all cases, reversible by removal of the inhibiting agent.

The present study was initiated as a search for irreversible inhibitors which could be used to label or chemically modify amino acids at the active center of invertase. A wide variety of group specific reagents is available. We concentrated on those specific for amino acids already implicated as active center residues, e.g. histidine [5], cysteine [3] and methionine [6]. We were, however, limited by the pH sensitivity [7] of invertase to those reagents which were effective in a pH range of 4.2 to 7.

Materials

All chemicals were reagent grade. Aniline was redistilled before use. Glucostat was purchased from Worthington Biochemical Corp. and radioactive iodide (^{125}I-labelled NaI) from New England Nuclear. Pyridoxal, fructose, and cysteine were purchased from General Biochemicals. Potassium iodide and iodine were obtained from J.T. Baker. Sucrose was purchased from Mallinkrodt Chemical Works and hydroxylamine from The Matheson Co. Mercaptoethanol was provided by Sigma Chemical Co. Eastman Chemical Co. was the source of bromoacetic acid and iodoacetic acid. 2-hydroxy-5-nitrobenzyl bromide and iodoacetamide were obtained from Pierce Chemical Co. *p*-Chloromercuribenzoate was purchased from Nutritional Biochemical Co.

Methods

Enzyme purification

Growth conditions for *Neurospora crassa* SF26 and procedures for invertase purification were described by Metzenberg [3] and Meachum et al. [1].

Assays

The invertase assay utilizing Glucostat was described by Meachum et al. [1]. For reactions with aniline, the reducing sugar assay of Nelson [8] was used to measure enzyme activity. Protein concentration was determined by the method of Lowry et al. [9] or by measuring the optical density at 280 nm [1]. Specific activity of invertase was expressed as μmoles of sucrose hydrolyzed per

mg of protein per min. Total hexose was measured by the phenol-sulfuric acid method of Dubois et al. [10], using glucose as the standard.

Inhibition studies

Reactions were carried out at pH 5 in 0.05 M acetate buffer unless otherwise noted. These conditions were always restored by dialysis before assaying for invertase activity. The volume of the enzyme-inhibitor reaction mixture was 1 ml or less and contained 1—2 mg purified invertase. Each inhibitor was tested two or more times; only the most strenuous conditions used for a particular reagent are reported.

Iodine inactivation of invertase

Invertase was treated with appropriate concentrations (15 to 100 molar excesses) of iodine as described under Table II. The specific activity of iodine-inactivated invertase was determined for each sample to confirm inactivation.

Sedimentation velocity analysis

The sedimentation coefficients of invertase and iodine-inactivated invertase in 0.1 M NaCl and acetate buffer were determined as described by Meachum et al. [1]. Samples were centrifuged at 20° C and 56 000 rev./min in a Beckman Spinco Model E analytical ultracentrifuge.

Fingerprints and one-dimensional electrophoresis

Tryptic digestion and fingerprinting were carried out according to the procedures of Helinski and Yanofsky [11]. The chromatography solvent consisted of pyridine-*n*-butanol-acetic acid-water (60 : 90 : 18 : 70, by vol.). The pH 3.5 buffer system used during electrophoresis consisted of pyridine-acetic acid-water (1 : 10 : 300, by vol.). Ehrlich's reagent was prepared and applied by the method of Easley [12]. High voltage electrophoresis was carried out on apparatus manufactured by Savant.

Results and Discussion

Effects of group specific reagents on invertase activity

The results of our survey show that *N. crassa* invertase is remarkably resistant to inhibition by the standard group specific reagents. Reactions with aniline, pyridoxal, sucrose and fructose under reducing conditions failed to inhibit invertase irreversibly through reduction of a Schiff's base (Table I). Enzyme substrate and product were included because the proposed [13] mechanism of invertase catalysis requires Schiff's base formation between a carbonyl group of the sucrose substrate (or fructose product) and an amino group of the enzyme. There was little or no inhibition; instead, fructose and sucrose appear to protect the enzyme against the effects of strenuous treatment with $NaBH_4$.

Tests with *p*-chloromercuribenzoate, bromoacetic acid, iodoacetic acid, iodoacetamide, hydroxylamine and 2-hydroxy-5-nitrobenzyl bromide were either negative or positive at levels of inhibition too low to be useful (Table II). The specificities of these reagents under the conditions employed are given in Table II.

TABLE I

INHIBITION BY SCHIFF'S BASE FORMING REAGENTS

Inhibition (%) is based on the specific activity of invertase in the control reaction for each inhibitor.

Reagent	Concentration (mol reagent/mol enzyme)	Inhibition (%)
Aniline*	2536	0
Aniline + $NaBH_4$*	2536	5
Pyridoxal**	4768	5
Pyridoxal + $NaBH_4$**	4768	20
Fructose***	22100	0
Fructose + $NaBH_4$***	22100	15
Sucrose***	22100	0
Sucrose + $NaBH_4$***	22100	0
$NaBH_4$†	—	41

* Aniline was allowed to react with invertase for 5 min at 25°C and dialyzed against 0.005 M $NaBH_4$ for 5 min at 0°C.

** Pyridoxal was adjusted to pH 4.5 and incubated 30 min at 0°C with invertase; some samples were dialyzed an additional 15 min against 0.05 M $NaBH_4$.

*** Invertase was dialyzed 1 1/2 h at 37°C against 0.05 M $NaBH_4$ containing 0.1 M sucrose or 0.1 M fructose. The pH of the fructose-containing dialysate was 6.9; that of the dialysate with or without sucrose was 9.2.

† Dialysis against 0.05 M $NaBH_4$ for 1 1/2 h at 37°C. Except for this example, employing strenuous conditions, $NaBH_4$ alone did not inhibit invertase.

TABLE II

INHIBITION BY GROUP SPECIFIC REAGENTS

Inhibition (%) is defined in Table I. Hydroxylamine and *p*-chloromercuribenzoate were incubated with invertase for 20 min at 25°C. Bromoacetic acid at pH 5.5 was reacted with enzyme for 3 h at 37°C. Bromoacetic acid, iodoacetic acid and iodoacetamide at pH 4.5 were incubated with invertase in the dark for 2 1/2 h at 37°C. 2-hydroxy-5-nitrobenzyl-bromide was dissolved in dry acetone, and concentrations were adjusted to give a final concentration of 5% acetone in the reaction mixture. The mixture was stirred vigorously for 2 min at 25°C. Iodine and potassium iodide were incubated with invertase for 10 min at 25°C.

Reagent	Specificity	Concentration (mol reagent/ mol enzyme)	Inhibition (%)
Hydroxylamine	Aspartate, glutamate [14]	2536	2
p-chloromercuribenzoate			
(pH 4.6)	Cysteine [14]	2.2	15
		20.0	1
(pH 5.0)	Cysteine [14]	2.2	0
		20.0	12
Bromoacetic acid			
(pH 5.5)	Cysteine, histidine [15]	65600	31
(pH 4.5)	Cysteine, methionine [16]	50	3
Iodoacetic acid	Cysteine, methionine [16]	50	0
Iodoacetamide	Cysteine, methionine [16]	50	15
2-hydroxy-5-nitrobenzyl bromide	Tryptophan [17]	1000	20
Iodine	Tyrosine, histidine, tryptophan, cysteine, methionine [14, 18]	22	96
Potassium iodide		11800	6

TABLE III

p-CHLOROMERCURIBENZOATE TITRATION OF CYSTEINE IN INVERTASE

Titrations were performed according to the procedure of Boyer [19] and Riordan and Vallee [20]. Titrated in 0.33 M acetate buffer at pH 4.6 and 0.05 M acetate buffer at pH 5.0. *p*CMB, *p*-chloromercuribenzoate.

Conditions	μmol *p*CMB per μmol invertase	mol cysteine per mol invertase
pH 4.6	3.8/2.1	1.8
pH 4.6 + 6 M urea	3.1/2.1	1.5
pH 5	2.8/2.1	1.3
pH 5	2.5/2.2	1.2
pH 5 + 6 M urea	4.2/2.1	2.0

The negative results with *p*-chloromercuribenzoate were surprising since titrations (Table III) of invertase indicated that 1—2 moles of this reagent are, in fact, bound under non-dissociating conditions, and that at most two moles of cysteine are present per mole of invertase.

Inhibition by iodine

Iodine, an inhibitor of yeast invertase [6,21], was the only reagent tested which irreversibly inhibited *Neurospora* invertase. The effect of iodine concentration on inactivation of invertase is shown in Fig. 1. As can be seen from the

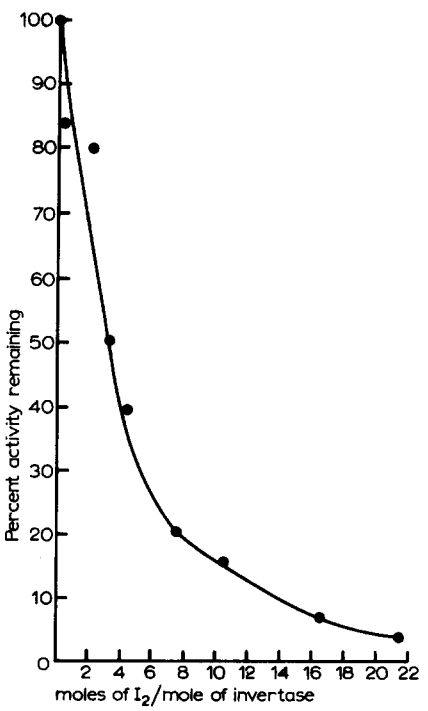

Fig. 1. Effect of concentration of iodine on inactivation of invertase. Specified concentrations of iodine in 0.1 M potassium iodide in 0.05 M acetate buffer (pH 5) were incubated with invertase for 10 min at 25°C. After dialysis against the acetate buffer, specific activity was determined.

TABLE IV

THE EFFECT OF SUBSTRATE ON IODINE INHIBITION

Invertase was incubated with iodine at a concentration of 16.5 moles of iodine per mol of invertase in the presence or absence of sucrose (17 mg/ml).

Reaction conditions	Invertase sample	Inhibition (%)
25°C, 10 min	+ sucrose	90
	− sucrose	91
0°C, 30 min	+ sucrose	77
	− sucrose	72

inhibition curve, 7—8 moles of iodine per mol of invertase were sufficient to produce 80% inhibition. This rapid inactivation by low concentrations of iodine indicates specific inhibition, through interaction with an active center residue. Prevention of inactivation by substrate is also good evidence of specificity but we were unable to demonstrate protection by substrate (Table IV).

The enzyme could not be reactivated by the thiols, mercaptoethanol or cysteine (Table V). This is in contrast to iodine-inactivated yeast invertase [6] which can be reactivated by mercaptoethanol, though not by cysteine.

Iodine may be very specific in its action on a particular protein, but it is capable of reacting with a number of amino acid residues. The reactions involve either oxidation or electrophilic substitution [22]. Tyrosine, histidine and, occasionally, half-cystine are iodinated; tryptophan, methionine and half-cystine are oxidized. Iodination is readily detected by the use of radioactive iodine. Experiments (Table VI) with radioactive iodine demonstrated that invertase is not iodinated under the conditions employed to inactivate the enzyme.

Physical and chemical characterization of iodine-inactivated invertase

Sedimentation velocity studies of iodine-treated invertase demonstrated that the quaternary structure of the enzyme was not altered and that no major conformational changes occurred as a result of iodine inactivation. The $S_{20,w}$ value of 10.45 S obtained with iodine-treated invertase correlates well with that for an untreated sample (10.35 S).

Tryptic digests of iodine-treated and untreated invertase were subjected to one-dimensional high-voltage electrophoresis and stained with Erhlich's reagent

TABLE V

EFFECT OF THIOLS ON IODINE-INACTIVATED INVERTASE

Following iodine treatment (11 mol iodine per mol invertase) of invertase, 0.23 M mercaptoethanol (26822 mol/mol enzyme) or 0.02 M cysteine (2332 mol/mol enzyme) was added to the reaction mixture and incubated 10 min at 25°C. After dialysis against acetate buffer, specific activity was determined.

Thiol	% Inhibition before thiol addition	% Inhibition after thiol addition
Mercaptoethanol	84	83
Cysteine	84	85

TABLE VIII

AMINO ACID COMPOSITION OF IODINE-INACTIVATED INVERTASE VERSUS UNTREATED INVERTASE

Samples (1 mg) of I_2-inactivated invertase and untreated invertase (control) were hydrolyzed under vacuum at 110°C for 48 h and dried under vacuum over NaOH pellets and sulfuric acid. These residues were dissolved in 1 ml of a pH 2. 2 dilution buffer and analyzed [23] on a Beckman Spinco Model 120 C Amino Acid Analyzer. Only values for altered amino acids and, for comparison, lysine, histidine, and aspartate are tabulated. Values are presented as number of residues per mol [1].

Amino acid	Number of residues		Residues of I_2-inactivated
	I_2-inactivated	Control	Residues of control
Lys	60.2	59.3	1.01
His	23.7	24.9	0.95
Asp	183.9	178.7	1.03
Tyr	55.5	65.8	1.03
Met sulfone	2.6	Trace	—

present in the experimental sample less the amount in the control represents the amount of sulfoxide produced by iodine treatment. The results (Table IX) showed no difference between experimental and control samples in methionine and methionine sulfone content. This modified analysis did show that more cysteic acid (43%) was present in iodine-inactivated samples and that S-carboxymethyl cysteine was present in control samples only. Again, tyrosine was lower in iodinated samples. Lowering of tyrosine in the non-iodinated control samples (compare Tyr in Tables VIII and IX) reflects the effect of performic acid treatment.

These results are difficult to interpret. The evidence from inhibitor concentration studies and from physical and chemical characterization of the inactivated enzyme demonstrates that the reaction is a selective one, probably involving an active center residue. Amino acid analyses indicate alteration of tyrosine and cysteine (half-cystine). However, the experiments on uptake of

TABLE IX

AMINO ACID COMPOSITION OF IODINE-INACTIVATED INVERTASE VERSUS UNTREATED INVERTASE AFTER CARBOXYMETHYLATION AND PERFORMIC ACID OXIDATION

Samples were treated according to the procedure of Neumann et al. [24] for detection of methionine sulfoxide and were performic acid oxidized at 0°C according to the procedure of Hirs [25].

Amino acid	Number of residues		Residues of I_2-inactivated
	I_2-Inactivated	Control	Residues of control
Lys	66.6	64.5	1.05
His	25.2	25.7	0.99
Asp	174.9	187.3	0.95
Cysteic acid	11.4	7.3	1.43
Met sulfone	7.1	6.8	1.07
Met	3.2	2.8	1.16
S-carboxymethyl Cys	0.0	0.8	
Tyr	38.6	53.9	0.73

TABLE VI

INCORPORATION OF RADIOACTIVE IODINE (^{125}I)

Radioactive iodide (^{125}I-labelled NaI) was mixed with 0.005 M iodine in 0.1 M KI and 0.05 M acetate buffer to give a specific activity of $7.46 \cdot 10^6$ cpm/μgatom iodine. For each reaction, 0.1 ml ^{125}I solution was added to 1.4 mg invertase in 0.1 ml acetate buffer. After 10 min incubation at 25°C, the reaction in some samples was stopped by addition of cysteine to a final concentration of 0.02 M. All samples were dialyzed against 0.05 M acetate buffer (pH 5). Protein concentration was determined and 0.2 ml aliquots were added to 9.8 ml scintillation cocktail (60 mg naphthalene, 4 mg PPO, 200 mg POPOP, 100 ml methanol and dioxane to 1 l). The samples were counted in a Beckman liquid scintillation system. Values are the average of three determinations.

Conditions	gatom I incorporated per mole of invertase
Reaction terminated by dialysis	0.36
Reaction terminated by addition of cysteine	0.03

which is specific for tryptophan. There was no difference in the electrophoretic mobilities of the tryptophan peptides of the control and the iodine-treated samples and no peptides were missing from the iodine-treated sample.

Fingerprints prepared from tryptic digests of iodine-treated and -untreated invertase did not reveal convincing differences between the two samples. The major spots appeared identical.

Neurospora invertase is a glycoprotein containing 11% mannose and 3% glucosamine [1]. Measurements of the amount of carbohydrate associated with the enzyme indicated that no loss of carbohydrate occurred during iodine inactivation (Table VII).

Standard amino acid analyses of iodine-treated invertase (Table VIII) revealed very few differences between experimental and control samples. There was some loss of tyrosine and a trace amount of methionine sulfone was present in the iodine-treated samples. This suggested oxidation of methionine, which has been proposed [6] as the mechanism of inhibition of yeast invertase by iodine. The procedure for amino acid analysis was modified [24] for detection of methionine sulfoxide, an unstable oxidation product of methionine. Samples were carboxymethylated and performic acid oxidized before hydrolysis. This is necessary because methionine is regenerated from methionine sulfoxide during acid hydrolysis. Methionine sulfoxide is resistant to carboxymethylation, so that only methionine will be carboxymethylated. Then, during performic acid oxidation, the methionine sulfoxide will be converted to methionine sulfone, which is stable to acid hydrolysis. The amount of sulfone

TABLE VII

THE EFFECT OF IODINE ON ENZYME CARBOHYDRATE

Invertase	mol I_2 per mol invertase	mg hexose per mg protein
Control	0	0.110
I_2-inactivated	21	0.138
	1099	0.125

radioactive iodine demonstrate that inactivation does not proceed through iodination of tyrosine or half-cystine. The chemistry of oxidation of amino acids by iodine in such a complex environment as a protein molecule is incompletely understood. This is particularly acute in the case of tyrosine. Complete oxidation of sulfhydryl compounds by iodine proceeds through a series of products (disulfide, sulfenyl iodide, sulfenate, sulfinate) to sulfonate. In the case of half-cystine, the final product is cysteic acid. Standard amino acid analyses show that cysteic acid is not produced by iodine inactivation of invertase. Thus, it is possible that an unstable oxidation product, such as sulfenic acid, is the product of interaction of half-cystine and iodine during invertase inactivation. Though it seems unlikely that such a highly unstable oxidation product could be maintained in the protein, recent research [26,27] suggests that the immediate environment of the sulfhydryl undergoing oxidation by iodine influences the extent of oxidation, as well as the stability of the product. For example [26], thiols which have a free carboxyl group on a carbon atom β or γ to the sulfur group show an increased tendency to overoxidize in the presence of iodine. It is interesting to note that there is evidence [6] that a carboxylate anion is in close proximity to the residue in yeast invertase being altered by iodine.

The apparent loss of tyrosine might also be explained by the presence of oxidized half-cystine. It is known [24] that losses of tyrosine during acid hydrolysis are particularly large when the protein contains methionine sulfoxide. Oxidized half-cystine may have the same effect on tyrosine during preparation of iodine-inactivated invertase for amino acid analysis.

In conclusion, we advance the hypothesis that *Neurospora* invertase is inactivated by iodine through oxidation of half-cystine to a normally unstable oxidation state which is maintained and protected by its protein environment and that loss of tyrosine may be an artifact caused by the presence of this residue during acid hydrolysis.

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