BBA 66362

CHEMICAL MODIFICATION OF THE ACTIVE SITE OF YEAST INVERTASE

A. WAHEED* AND S. SHALL
Biochemistry Laboratory, University of Sussex, Brighton BN1 9QG, Sussex (Great Britain)

(Received December 21st, 1970)**

SUMMARY

1. Reaction of iodine with yeast invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) gives a stable product with about half the enzyme activity. This inactivation is not reversed by cysteine but is reversed by mercaptoethanol or mercaptoethylamine. The kinetics of the reactivation show it to be first order in both iodine–invertase and mercaptoethanol. pH affects the reactivation process in a complex way; two groups on the protein affect the rate. One with a pK_a of about 6.2 enhances the rate in its protonated form, while the second with a pK_a of about 8.0 is required in the base form.

2. A comparison of the kinetics of invertase and iodine–invertase reveal that the catalytic constant ($k + 2$) in the latter is almost half the value in invertase. The pK_a value of the group(s) affecting the catalytic constant is not altered by the iodine reaction. There is some evidence that two acid groups are involved; a histidine residue with a pK_a of 6.8–6.9 and a residue with a pK_a of 8 or greater. The K_m value of iodine–invertase is possibly increased above the value for invertase.

3. Iodotyrosine is not formed in the iodine reaction. Nitration of tyrosines in invertase does not affect enzyme activity.

4. Iodoacetamide inhibits yeast invertase, but iodoacetic acid does not. The inhibition reaction is first order in enzyme and in iodoacetamide. The effect of pH on the inhibition is complex; a group with a pK_a of 7.0 enhances the reaction in its protonated form. The group that is alkylated has a pK_a well in the alkaline region. The iodine reaction and the iodoacetamide inhibition are quite independent reactions.

5. CNBr inhibits yeast invertase activity in two separate reactions. The first, fast reaction is dependent on pH. The second, slow reaction is independent of pH and is probably at a methionine sulphur atom. The iodine reaction and the inhibition by CNBr are independent reactions.

6. Methyl mercury nitrate inhibits yeast invertase. The 50% inhibition concentration is $1.2 \cdot 10^{-6}$ M; the inhibition therefore probably involves a chelate complex of the active site histidine and another atom, possibly a sulphur of a methionine.

7. The hypothesis is advanced that the iodine reaction is the reversible oxidation of a methionine sulphur atom and that this leads to half the number of effective

* Present address: Laboratory of Molecular Biology, Department of Medicine, State University of New York, Brooklyn, N.Y. 11203, U.S.A.

** Publication delayed due to the British postal strike.

active sites. It is postulated that the active site contains a carboxylate anion because of the difference in reactivity between cysteine and mercaptoethylamine, and between iodoacetamide and iodoacetic acid. On the basis of the observations a reasonable mechanism of action of yeast invertase is postulated.

INTRODUCTION

Low concentrations of iodine inhibit yeast invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) in a peculiar and characteristic way. VON EULER AND LANDERGREN[1] who first described this phenomenon in 1922 observed that an inhibition of about 55% occurred, when invertase was reacted with iodine solutions. The resulting product is called iodine–invertase (I–invertase), without in any way indicating the nature of the reaction. Later it was reported that the K_m is not altered nor is the activity–pH curve[2–4]. The nature of I–invertase is unknown. The fact that I–invertase is insensitive to silver and mercury ions in concentrations that inhibit the native enzyme completely, led MYRBÄCK[5] to suggest that reaction occurred with –SH groups. In the same review, MYRBÄCK[5] drew attention to the fact that reactivation of I–invertase by –SH compounds or other reducing agents had not proved possible[4]. The reaction of iodine and invertase is not pH dependent. Both bromine and chlorine produce complete inactivation of the enzyme; thus there is a degree of specificity about the iodine reaction.

We have reinvestigated the reaction between iodine and external invertase. The partially inhibited enzyme can be reactivated by reducing agents of appropriate nature. Similarly, we have examined the inhibition of invertase by iodoacetamide and iodoacetic acid. Here, too, an unexpected difference in reactivity was observed. The reaction between cyanogen bromide and invertase was found to consist of two different inhibiting reactions. The nature of these several reactions are discussed and their contribution to understanding the active site of invertase is outlined. Some of this work has been reported in a preliminary form[6,7].

External yeast invertase is a glycoprotein containing about 50% carbohydrate[25,27]. Internal invertase is found inside the yeast cell[26,27] and contains no carbohydrate. The two enzymes are reported to differ in amino acid composition[22]; in particular, the internal enzyme does not contain cysteine. The molecular weight of external yeast invertase is 270 000 (ref. 25) and therefore the molecular weight of the protein portion is about 135 000.

MATERIALS

DEAE-Sephadex and Sephadex G-200 were from Pharmacia Ltd. Iodacetamide and iodoacetic acid were recrystallised from light petroleum ether after decolourisation with charcoal. Only colourless, white material was used.

CNBr was purchased from Eastman Kodak Ltd.

Tetranitromethane was purchased from Koch-Light Laboratories Ltd.

Methyl mercury nitrate was prepared from methyl mercury iodide.

Methyl mercury iodide was prepared by the reaction of methyl iodide and metallic mercury. 25 g of methyl iodide was shaken with 36 g of mercury in a 200 ml

round bottomed flask for 24 h during which time it was continuously irradiated with a low pressure ultraviolet lamp. Over the period the mixture solidifies to a yellow mass.

$$CH_3I + Hg \rightarrow CH_3HgI$$

The crude methyl mercury iodide was extracted with methanol, filtered, concentrated under reduced pressure and recrystallised from methanol to yield white flakes.

The iodide is displaced by silver nitrate to yield methyl mercury nitrate.

$$CH_3HgI + AgNO_3 \rightarrow CH_3HgNO_3 + AgI$$

3 g of methyl mercury iodide were dissolved in 10 ml of methanol in a 200-ml stoppered round bottomed flask. 1.47 g silver nitrate was added and the mixture was shaken for 30 min during which time a heavy, yellow precipitate of AgI formed. The solution was filtered and the precipitate washed 3 times with 10 ml of methanol. The methanol extracts were dried under vacuum and recrystallised from methanol.

Buffers were prepared from sodium acetate, pH 4.0 to 6.0, sodium phosphate, pH 6.0 to 8.0, or sodium borate, pH 8.6 to 9.2. All other chemicals were Analytical Reagent Grade.

METHODS

Ultraviolet absorption spectra were recorded with a Cary Model 15 recording spectrophotometer. pH was measured in a Pye Model 79 pH meter which was routinely calibrated with standard buffer solutions at pH 4.00 and 7.00.

Carbohydrate content was estimated by the method of MORRIS[28] as modified by CHUNG AND NICKERSON[29]. Glucose was used as a standard and it was assumed that the polysaccharide gave the same colour yield as glucose.

Iodine was dissolved in equimolar solutions of KI; 1.0 mM iodine solution contained 1.0 mM KI.

Enzyme assay. (1) The assay solution contained 2.0 ml of 0.10 M sucrose in 0.10 M sodium acetate buffer, pH 4.9, at 25.0° and 0.10 ml of a suitable dilution of the enzyme solution. After a suitable time interval, usually 15 min, 0.10 ml was removed into copper sulphate solution for reducing sugar estimation. The enzyme reaction was stopped by the copper sulphate. The concentration of reducing sugars (glucose and fructose) formed by the hydrolysis of sucrose was estimated by NELSON'S[8] method. A unit of enzyme activity is defined as the amount of enzyme which hydrolyses 1.0 μmole of sucrose per min at 25° in 0.10 M sodium acetate buffer, pH 4.9. (2) Polarimetric assay: 0.10 ml of enzyme was mixed with 2.0 ml of 0.10 M sucrose in acetate buffer, pH 4.9, at 25°. The optical rotation of the solution was recorded every minute in a Perkin–Elmer Model 141 M polarimeter at the sodium D line and at 25°. The linear initial portion of the graph was used to determine the rate of change of optical rotation.

Preparation of enzyme. Yeast invertase concentrate was purchased from British Drug Houses Ltd. The enzyme concentrate is obtained from *Saccharomyces cerevisiae.* All operations were carried out in the cold room (0–4°).

1 l of invertase concentrate was thoroughly dialyzed for 48 h against 5 mM sodium acetate buffer, pH 5.0, to remove glycerol and other low molecular weight

impurities. The solution (2170 ml) was heated for 30 min at 50° in a water bath and cooled rapidly. The precipitate was removed by centrifugation at 5000 × *g* for 15 min. The clear supernatant liquid (2060 ml) contained all the enzyme activity; this was precipitated by the addition of an equal volume of 95% ethanol which had been precooled to −20°. The solution was allowed to stand for 10 min in the cold room and was then centrifuged for 10 min at 10 000 × *g*. The precipitate was redissolved in 0.10 M sodium acetate buffer, pH 4.9. This enzyme solution was then dialyzed against 0.010 M sodium phosphate buffer, pH 7.0, followed by chromatography on DEAE-Sephadex at pH 7.0. The chromatography was on a 24 cm × 2.5 cm column of DEAE-Sephadex A50 equilibrated with 10 mM phosphate buffer, pH 7.0. The enzyme was eluted by a gradient of NaCl, 0 to 0.150 M. The overall yield of enzyme activity was 49% and the enzyme was purified 4.5 times. The pure material was homogenous on polyacrylamide gel electrophoresis[9] at pH 8.6 and 6.5; it consisted of about 50% carbohydrate and the nitrogen content was estimated to be 6.63%. The specific enzyme activity of the pure material was 2161 units/mg of protein at 25°. This pure enzyme is the external yeast invertase[25,27] and not the internal enzyme[26,27,22].

For kinetic analysis the assays were performed as described above, except that the concentration of sucrose was varied at each pH value. The kinetic parameters K_m and v_{max} were estimated by linear regression on the reciprocal of the Michaelis–Menten equation, followed by reiteration on the original equation until a constant value of the parameters was achieved. The computations were done with a computer program kindly given by Professor W. W. CLELAND[10] and slightly modified in two respects. We established experimentally that the variance of the mean of an experimentally determined velocity varied linearly with the square of the value of the velocity. Therefore, we weighted each velocity with the square of that velocity for the normal equation and with $1/v^4$ for the reciprocal form. We also calculated the coefficient of the variance defined as the ratio of the standard error to the mean value. The value of the constant k_{+2} was estimated by dividing the estimated v_{max} value by the measured enzyme concentration.

RESULTS

Reaction between invertase and iodine

The reaction between iodine and purified yeast invertase (1.1 μg/ml, approx. 3 nM) was examined at several iodine concentrations (0.02 to 1.0 mM). Iodine inhibits the activity of yeast invertase. The reaction was extremely rapid and was complete within 2 min. Observation for a further 30 min revealed no further reaction. Similar results were obtained at all the iodine concentrations tested. More striking, the extent of the reaction was partial, only about half the enzyme activity was lost. The maximum loss required only 0.1 mM iodine (Fig. 1). The product of the reaction will be designated iodine-invertase without presupposing in any way the nature of the reaction.

Reactivation of iodine–invertase

MYRBÄCK AND WILLSTAEDT (1958)[4] reported that the enzyme activity of iodine–invertase could not be regenerated by reaction with such reducing compounds as cysteine or ascorbic acid. None of the compounds they tested reactivated the enzyme.

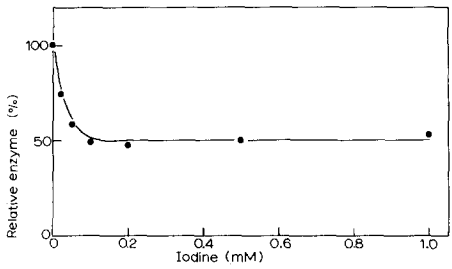


Fig. 1. Partial inactivation of yeast invertase by iodine. 5 ml of invertase (1.1 μg/ml, approx. 3 nM) in 0.1 M acetate buffer, pH 4.9, was reacted with iodine dissolved in KI. After 2, 10 and 30 min samples were removed for enzyme assay. There was no change in activity between 2 and 30 min.

We have confirmed that neither cysteine nor ascorbic acid at 0.10 M concentration can reactivate iodine–invertase. The light yellow colour of the remaining iodine disappeared immediately upon the addition of the reducing agent, but no return of enzyme activity occurred.

On the other hand, we have observed that enzyme activity can be restored if suitable reducing reagents are used. In particular, mercaptoethanol and mercaptoethylamine are able to reverse the inhibition brought about by reaction with iodine.

A representative result with mercaptoethanol is shown in Table I. 90% of the original enzyme activity was restored by treating the partially inactive iodine–invertase with mercaptoethanol. In this experiment it was found, of course, that the mercaptoethanol interfered with the reducing sugar determination. For this reason, mercaptoethanol was added in equal concentration to the control solutions. To further confirm that the reactivation was not spurious, we repeated the experiment using the polarimeter to assay enzyme activity. This experiment, confirmed the first experiment precisely. This confirmation together with the kinetics of reactivation described below, establish the ability to reactivate iodine–invertase by reaction with mercaptoethanol.

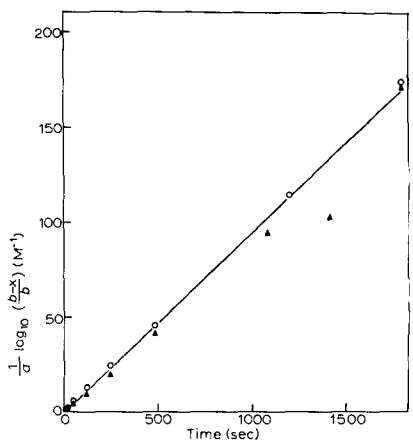
TABLE I

REACTIVATION OF IODINE–INVERTASE BY MERCAPTOETHANOL

Enzyme activity was assayed by estimation of reducing sugar.

	Enzyme activity (units)	*Relative enzyme activity (%)*
1. Control: invertase	2200	100
2. Iodine–invertase	1100	50
3. Mercaptoethanol-treated iodine–invertase	1980	90

2.7 μg of pure invertase in 4.5 ml of acetate buffer, pH 4.9, 25° was used. 100 μl was taken for enzyme assay; this was control activity. To the remaining 4.4 ml was added 0.5 ml of 1 mM iodine in KI. The solution was mixed and after 5 min 100 μl aliquot taken for activity measurement; this was designated iodine–invertase. The remaining solution (4.8 ml)was made 100 mM in mercaptoethanol and after 5 min a 100 μl aliquot was taken for enzyme activity determination.



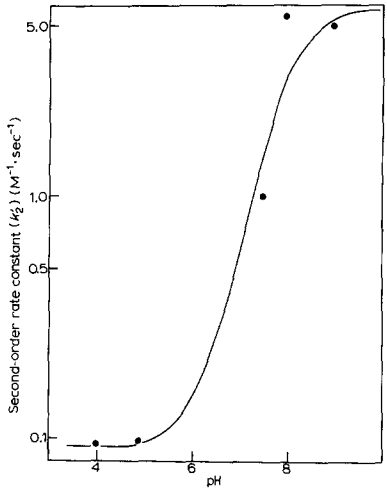


Fig. 2. Kinetics of the reactivation of partially inactive iodine–invertase by mercaptoethanol. Iodine–invertase (1.4 μg/ml) was reacted with either 2.5 or 5.0 mM mercaptoethanol in 0.1 M acetate buffer, pH 4.0, 25°. Samples were removed at intervals for enzyme assay. a is the concentration of mercaptoethanol present in large excess and therefore of constant concentration, b is the concentration of iodine–invertase and $(b - x)$ is the concentration of reaction product, that is, reactivated invertase. ○, 2.5 mM; ▲, 5.0 mM mercaptoethanol. The slope of the line gives the second-order rate constant.

Fig. 3. The variation of the rate of reactivation of iodine–invertase by mercaptoethanol with pH The vertical ordinate shows the second-order rate constants (k'_2), $M^{-1}\cdot sec^{-1}$, established as in Fig. 2, on a logarithmic scale. The points are experimental. The line is drawn from Eqn. 6, where k_2 is a pH-independent rate constant, K_1 and K_2 are dissociation constants of acids and H is the H^+ concentration. The line arises from the values $k_2 = 5.9\ M^{-1}\cdot sec^{-1}$; $pK_1 = 6.20$; $pK_2 = 8.00$.

The kinetics of the reaction between iodine–invertase and mercaptoethanol were studied at several pH values at 25°. It was observed that the reappearance of enzyme activity followed a simple first-order rate law for both enzyme activity and mercaptoethanol (Fig. 2). Consequently, the data are plotted as second-order reactions, using two concentrations of mercaptoethanol. Fig. 2 shows the data for pH 4.0 plotted from the equation,

$$k_2 \cdot t = \frac{1}{a} \log \frac{(b - x)}{b} \tag{1}$$

which is simply derived from the second order rate equation,

$$k_2 = \frac{1}{t} \frac{1}{(a - b)} \log \frac{a(b - x)}{b(a - x)} \tag{2}$$

by assuming that at all times $a \gg b$, where a is the concentration of mercaptoethanol, b is the concentration of iodine–invertase, $(b - x)$ is the concentration of the reaction product, that is, reactivated invertase.

It is clear from Fig. 2 that the data fit a second-order rate law satisfactorily. The rate of reactivation was measured at pH 4.0, 4.9, 7.5, 8.0 and 9.0. The lines were obtained by regression. A plot of the logarithm of the second-order rate constant against pH is shown in Fig. 3.

TABLE II

K_m VALUES ± STANDARD ERRORS OF INVERTASE AND IODINE–INVERTASE

pH	*Invertase (mM)*	*Iodine–invertase (mM)*	*Ratio of K_m of invertase to iodine–invertase*
4.9	18.0 ± 2.92	24.3 ± 2.36	0.74
6.0	29.5 ± 5.34	49.4 ± 9.53	0.60
6.8	14.7 ± 1.84	18.1 ± 2.79	0.81
7.5	13.3 ± 3.65	33.8 ± 9.30	0.39
8.0	17.3 ± 1.23	32.5 ± 3.16	0.53
9.0	21.1 ± 3.33	25.2 ± 3.47	0.84

In addition to mercaptoethanol, we found that mercaptoethylamine could also reactivate iodine–invertase. Invertase inactivated to 62% with iodine increased its activity to 86% following 5 min in 0.1 M mercaptoethylamine.

Stability of iodine–invertase

We have found the enzyme activity of iodine–invertase to be stable at room temperature. It could be reactivated by mercaptoethanol after 39 h at room temperature. Iodine–invertase is slightly less stable than invertase, see the lower part of Table III. Both forms of the enzymes are, however, relatively stable.

Kinetics of iodine–invertase

The enzyme activity of iodine–invertase was examined at several pH values, and the kinetic parameters K_m and k_{+2} were determined.

The K_m values for sucrose with iodine–invertase and invertase are shown in Table II. The data show large standard errors in every case. However, the ratio of

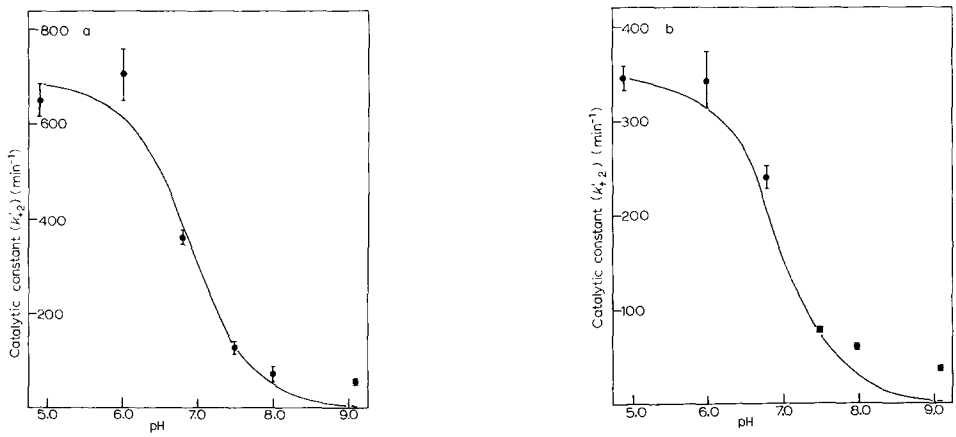


Fig. 4. The variation of the catalytic constant (k'_{+2}) of invertase (a) and iodine–invertase (b) with pH. The points are experimental; the vertical bars indicate 1 standard error above and below the mean. The lines are theoretical; the full lines are drawn from Eqn. 3, where k_{+2} is a pH-independent constant, K_a is the dissociation constant of an acid group in the enzyme substrate complex, H is the H^+ concentration and k'_{+2} is the constant at a given pH. The values used were: k_{+2}, for invertase of 693 min^{-1} and for iodine–invertase 352 min^{-1}; pK_a = 6.9.

the K_m values of invertase to iodine–invertase is less than 1.0 at all six pH values. It suggests that in fact there is a small increase in the value of K_m in iodine–invertase. This observation is substantiated in studies of internal yeast invertase where the increase in K_m value is clearly demonstrated[11,12].

The values of k_{+2} in invertase and iodine–invertase show a significant and reproducible difference (Fig. 4). The values of k_{+2} for iodine–invertase were lower at all the pH values examined. The ratios of the k_{+2} values varied from 0.51 to 0.78, and tend to be slightly higher in the more alkaline solutions. However, there is no substantial shift of the plot of k_{+2} against pH (Fig. 4); therefore, the pK_a value of the group responsible for this curve has not been substantially altered.

In Fig. 4a are shown the experimental values of k'_{+2} for external yeast invertase and Fig. 4b shows the same plot for iodine–invertase. The solid lines are drawn from Eqn. 3.

$$k'_{+2} = \bar{k}_{+2}/\left(1 + \frac{K_a}{H}\right) \tag{3}$$

This equation has been derived on the hypothesis that there is a single acid group in the enzyme–substrate complex that must be protonated for enzyme activity, as shown in Scheme 1.

$$\begin{array}{ccc} & \overset{k_{+1}}{\rightleftharpoons} & \\ E + S & & E\text{–}S \\ \Big\| K_b & \underset{k_{-1}}{} & \Big\| K_a \\ EH + S & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & EHS \xrightarrow{\bar{k}_{+2}} E + P \end{array}$$

$\bar{k}_{+2}$ is a pH-independent rate constant. K_b and K_a are acid dissociation constants of the group in the free enzyme and in the enzyme–substrate complex, respectively, and may be identical in value; the binding of the substrate may not alter the pK_a value. WALEY[16] has derived the equations for this scheme and Eqn. 3 shows the relationship between k'_{+2} estimated at the H^+ concentration H, and the constants $\bar{k}_{+2}$ and K_a.

The numerical values that generate the graphs in Figs. 4a and 4b are $\bar{k}_{+2}$ for invertase 693 min^{-1} and for iodine–invertase 352 min^{-1}; pK_a 6.9. The same pK_a value is used in both graphs. The observed k'_{+2} for invertase at pH 9.1 is greater than the simple assumption of one ionizing group predicts. In the case of iodine–invertase the observed values at pH 8.0 and 9.1 are also greater than predicted.

In an attempt to define more closely the nature of the reaction of iodine with invertase we examined some of the residues that might react with iodine. Iodine has a 2-fold reactivity and may iodinate residues like tyrosine or histidine or may oxidise cysteine, tryptophan[23], or methionine[24].

Tyrosine residues in invertase

We first examined the possibility that the iodine gave rise to iodotyrosines. Since iodotyrosines have pK_a values near 7.0 and in alkaline solution have absorption maxima near 310 nm it is possible to distinguish between tyrosine and iodotyrosines by comparing the absorption spectra at 310 nm in mildly alkaline solution. The pK_a

of tyrosine is near 10.0 and the absorption maximum in alkali is at 295 nm. The ultraviolet absorption spectra of equimolar solutions of invertase and of iodine–invertase were compared at pH 6.0, 7.8 and 8.6. The spectra of the invertase and iodine–invertase were identical at all three pH values indicating that iodotyrosines were not formed.

Further evidence to support this conclusion was obtained by nitrating invertase with tetranitromethane, which has frequently been used for the nitration of proteins[13,14]. When a protein is treated with tetranitromethane at pH 8.0, 3-nitrotyrosine is formed which has an absorption peak at 428 nm.

13.5 mg of invertase (0.05 μmole) were reacted with 250 μmoles of tetranitromethane in 2.0 ml Tris buffer, pH 8.0, 20°. After 1 h the reaction mixture was dialyzed thoroughly and the presence of nitrotyrosine was sought by measuring the absorption spectrum of the product at pH 7.2, 8.0 and 10.5. The treated protein showed an appreciable peak of absorbance at 428 nm indicating the presence of nitrotyrosine. However, there was no loss of enzymic activity. Consequently, we conclude that modification of tyrosine residues in invertase does not inhibit enzyme activity.

Reaction of invertase with iodoacetamide

To determine whether iodoacetamide could alkylate yeast invertase and inhibit it, we reacted purified yeast invertase with 100 mM iodoacetamide at pH 7.5 and 25° and measured the disappearance of enzyme activity with time.

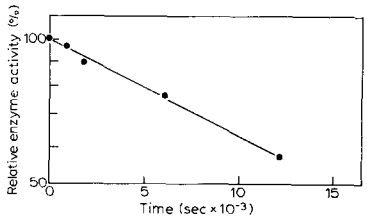


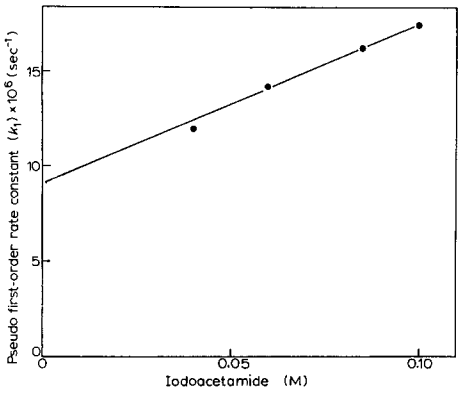
Fig. 5. The rate of the reaction between invertase and iodoacetamide. Invertase (1 mg/ml) was reacted with 0.1 M iodoacetamide at pH 7.5 in phosphate buffer, 25°. Aliquots were removed at intervals for enzyme assay. The vertical ordinate is logarithm of relative enzyme activity. The slope of the line is the pseudo first-order rate constant and was found by regression.

Iodoacetamide will react with a number of nucleophiles in the enzyme, but because we measure the reaction by its effect on enzyme activity we observe only those groups which are involved in the enzyme activity. Even here, of course, the reagent may be reacting with more than one group.

Iodoacetamide inhibited enzyme activity. The plot of the logarithm of the enzyme activity against time at a fixed concentration of iodoacetamide was linear (Fig. 5); the reaction is first-order in enzyme.

Since the logarithm of the enzyme activity decreases linearly with time, we can conclude that either only one group is involved in the loss of enzyme activity or that if more than one group is involved they are reacting at about the same rate with this reagent.

Consequently, we measured the pseudo first-order rate constant at four concentrations of iodoacetamide ranging from 40 to 100 mM at pH 7.5. From these experi-



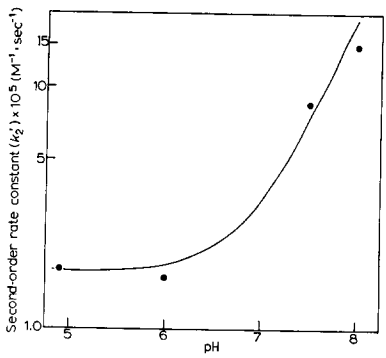


Fig. 6. The second-order rate constant for the reaction between invertase and iodoacetamide. The pseudo first-order rate constants for the reaction between invertase (1 mg/ml) in phosphate buffer, pH 7.5, at 25° and iodoacetamide were determined as shown in Fig. 5. These are plotted against the iodoacetamide concentration used. The slope of the resulting straight line (found by regression) yields the second-order rate constant.

Fig. 7. The variation in the rate of the reaction between invertase and iodoacetamide with pH. The second-order rate constants were estimated as shown in Fig. 6. The rate constant is plotted on the vertical ordinate on a logarithmic scale. The points are experimental. The line is drawn from Eqn. 4 where k'_2 is the rate constant at a given pH, $\bar{k}_2$ is a pH-independent rate constant, K_a is an acid-dissociation constant and H is the H^+ concentration. The values used are: $\bar{k}_2 = 1.70 \cdot 10^{-5}$ $M^{-1} \cdot sec^{-1}$ and $pK_a = 7.0$.

ments we calculated the four separate pseudo first-order rate constants (Fig. 6). By regression, we obtained the straight line of best fit, the slope of which gives the second-order rate constant for the reaction between yeast invertase and iodoacetamide at 25° and pH 7.5. The value of this constant was estimated to be $8.39 \cdot 10^{-5}$ $M^{-1} \cdot sec^{-1}$ with a standard error of $0.61 \cdot 10^{-5}$ $M^{-1} \cdot sec^{-1}$. Further, the reaction between invertase and iodoacetamide was investigated at pH 4.9, 6.0 and 7.8. Inhibition of enzyme activity was found at all three pH values. The pseudo first-order rate constants were estimated to be $1.78 \cdot 10^{-6}$ sec^{-1}, $1.64(\pm 0.27) \cdot 10^{-6}$ sec^{-1} and $14.8(\pm 0.84) \cdot 10^{-6}$ sec^{-1}, respectively. In all three experiments the iodoacetamide concentration was 100 mM. From this, second-order rate constants can be calculated (Fig. 7), assuming that the reaction is second-order at these pH values as it was shown to be at pH 7.5. A plot of pH against the logarithm of the second-order rate constant (k'_{+2}) is shown in Fig. 7.

The points are experimental, the line is theoretical. From pH 5 to 6 there is no increase in the rate constant, but from pH 6.0 to 8.0 there is a steady increase in the rate constant as anticipated for reaction of iodoacetamide with an acid which can be deprotonated over this pH range to release the reactive base. The line is drawn from the equation

$$k_2' = \bar{k}_2 \left(1 + \frac{K_a}{H}\right) \tag{4}$$

which has been previously utilised by HOUGH AND SHALL[15] and is analagous to the equation originally derived by WALEY[16]. $\bar{k}_2$ is a pH-independent rate constant, k'_2 is the observed rate constant at the H^+ concentration H, and K_a is an acid-

dissociation constant. The numerical values used in Fig. 7 are $\bar{k}_2 = 1.70 \cdot 10^{-5}$ $M^{-1} \cdot sec^{-1}$, and $pK_a = 7.0$.

In contrast to iodoacetamide, it was observed that 0.1 M sodium iodoacetate did not inactivate invertase at pH 7.8, 8.0 or 9.0 and 25° even after 325 min. After reaction with sodium iodoacetate at pH 7.8 the enzyme was still inhibited by iodine (to 61% activity) and reactivated by mercaptoethanol (to 95%).

Prior reaction of invertase with iodine to yield partially active iodine–invertase does not affect the reaction of the protein with iodoacetamide. The rate of the reaction of iodine–invertase with iodoacetamide was measured at pH 6.0. The calculated pseudo first-order rate constant was $1.92(\pm 0.71) \cdot 10^{-6}$ sec^{-1} compared to $1.64(\pm 0.27) \cdot 10^{-6}$ sec^{-1} for invertase at this pH; in both cases the concentration of iodoacetamide was 0.10 M.

In the reverse case, too, the two reactions appear to be independent. Invertase was reacted with 0.10 M iodoacetamide for either 8 or 23 h, the relative enzyme activity being 0.81 and 0.73. The two preparations were then treated with 0.1 mM iodine in KI and the fractional activity decreased to 0.46 (57%) and to 0.41 (56%), respectively. Invertase that had been reacted with 0.1 M iodoacetamide at pH 7.8 to an activity of 0.52 was treated with 0.1 mM iodine in KI. The fractional activity decreased to 0.30 (58%).

Amino acid analysis of invertase reacted with iodoacetamide showed the presence of *S*-carboxymethylcysteine, *N-im.*-carboxymethylhistidine and *N*-carboxymethyllysine.

Reaction of invertase with CNBr

In acid media CNBr reacts only with methionine and cysteine[17]; in alkaline media, of course, it will react with all available nucleophiles. Accordingly, we have reacted invertase with CNBr at both alkaline and acid pH. Invertase (1 mg/ml) was reacted with varying concentrations of CNBr at 25°, pH 8.0 and aliquots were removed after 20 min for enzyme assay. (Fig. 8). The graph consists of two portions. At low concentrations of reagent there is a sharp fall in enzyme activity, but above 1 mM concentration the slope of the line is much lower. The more reactive group gave only 50% inhibition. The results may be explained by the presence of two groups of quite different reactivity. Alternatively, the reagent may be destroyed under these conditions. To eliminate this latter possibility we reacted invertase with 20 mM CNBr at 25° and pH 8.0 for 20 min. We then assayed for enzyme activity and added further

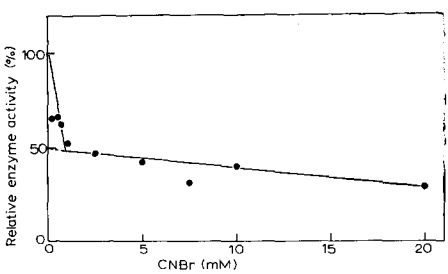
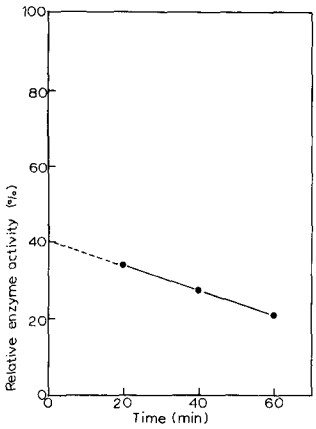


Fig. 8. Inactivation of yeast invertase by CNBr. Invertase (1 mg/ml) was reacted with CNBr at pH 8.0 in Tris buffer, 25°. After 20 min aliquots were removed for enzyme assay.



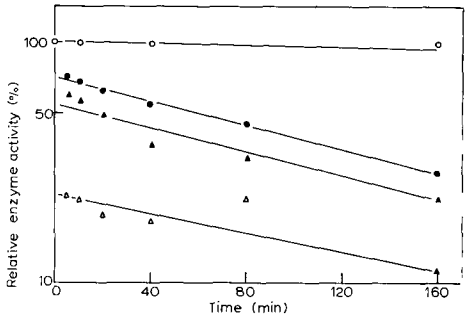


Fig. 9. Inactivation of invertase by CNBr: effect of adding fresh reagent. Invertase (1 mg/ml) was reacted with 20 mM CNBr in Tris buffer, pH 8.0, 25°. After 20 min an aliquot was removed for enzyme assay and fresh CNBr was added to a final concentration of 40 mM. At 40 min this was repeated to give a final concentration of 60 mM.

Fig. 10. The kinetics of the reaction between yeast invertase and CNBr. Invertase (1 mg/ml) was reacted with 0.1 M CNBr; samples were removed at intervals for enzyme assay. ●, pH 4.0; ▲, pH 6.0; △, pH 8.0; ○, control, no reagent, pH 8.0. The lines, found by regression, show only the slow CNBr reaction. Extrapolation back to zero time shows the extent of the fast reaction.

reagent to a total reagent concentration of 40 mM; at 40 min the addition was repeated to give a total concentration of 60 mM. The results (Fig. 9) show clearly that the more reactive processes went to completion with the first aliquot of reagent and the further additions only revealed the slower reaction.

Further evidence of the biphasic character of the reaction was revealed when the kinetics and the pH dependence of the reaction were examined. The kinetics of the reaction were measured at pH 4.0, 6.0 and 8.0 and 25° with 0.100 M CNBr (Fig. 10). It is immediately apparent that the enzyme inhibition consists of a fast reaction and a slow reaction. The fast reaction was complete at the first measurement at 5 min and its rate could not be measured. However, enzyme activity which was lost in the fast reaction was measured and was clearly pH dependent; the loss of activity

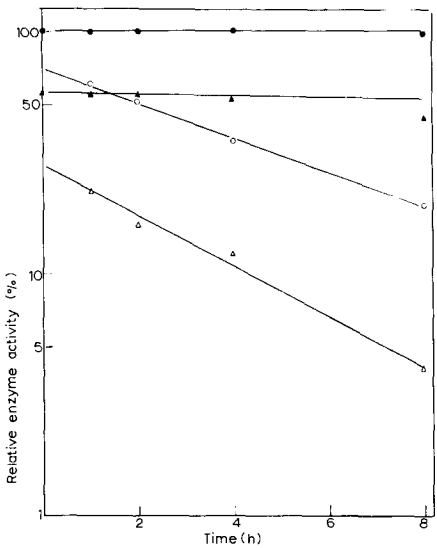
TABLE III

PSEUDO FIRST-ORDER RATE CONSTANTS AND THEIR STANDARD DEVIATIONS FOR THE REACTION BETWEEN CNBr AND INVERTASE OR IODINE–INVERTASE

pH	*Enzyme*	*Rate constant* (k_1) (sec^{-1}) × 10^5
4.0	Invertase	4.21 ± 0.15
6.0	Invertase	4.77 ± 0.87
8.0	Invertase	2.85 ± 0.89
4.9	Invertase	2.25 ± 0.23
4.9	Iodine–invertase	3.45 ± 0.56
Controls are rates of spontaneous loss of activity		
9.0	Invertase	0.09 ± 0.17
4.9	Invertase	0.046 ± 0.069
4.9	Iodine–invertase	0.37 ± 0.050

due to the fast reaction, increases with increasing pH; at pH 4.0 there is 30% inhibition and at pH 8.0 there is about 75% inhibition. The slow reaction is seen to be first order in enzyme. The pseudo first-order rate constants for the three pH values are listed in Table III. The rate of the slower reaction is not pH dependent; the rates at pH 4.0, 4.9, 6.0 and 8.0 are not significantly different. The weighted average of these four rates is $3.65 \cdot 10^{-5}$ sec^{-1}.

The reaction of iodine–invertase with CNBr was examined at pH 4.9 (Fig. 11). This derivative behaves like native invertase. Again, the initial fast reaction occurred



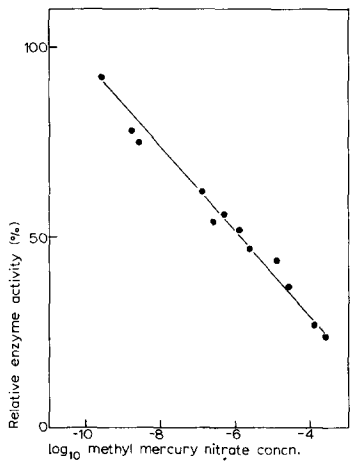


Fig. 11. Inhibition of invertase and iodine–invertase by CNBr. Protein (1 mg/ml) was reacted with 0.1 M CNBr in acetate buffer, pH 4.9, 25°. Aliquots were removed at intervals for enzyme assay. Invertase: ●, control; ○, with CNBr. Iodine-invertase: ▲, control; △, with CNBr.

Fig. 12. Inhibition of yeast invertase by methyl mercury nitrate.

in addition to the inactivation caused by the iodine. The fast reaction caused a drop to about 70% with native invertase and to about 28% with iodine–invertase; the iodine–invertase activity therefore decreased to about 60% of its original activity. The slower reaction is slightly faster with iodine–invertase, but this is probably accounted for by slightly greater instability of iodine–invertase.

Inhibition of invertase by methyl mercury nitrate

Invertase (1 mg/ml) was incubated with varying concentrations of methyl mercury nitrate and the degree of inhibition of enzyme activity was measured. A plot of relative enzyme activity against the logarithm of methyl mercury nitrate concentration is shown in Fig. 12. 50% inhibition occurred at $1.2 \cdot 10^{-6}$ M methyl mercury nitrate. This value is much higher than is expected for reaction with –SH groups, where a value of 10^{-12} M might be expected.

DISCUSSION

We have confirmed, once again, that dilute iodine can cause partial inactivation of yeast invertase. The inhibition reaction is extremely rapid; 3 nM enzyme is completely reacted by 100 μM iodine within 2 min, and it is apparent from Fig. 1 that under these conditions the reaction has gone to completion. The nature of the reaction is of considerable interest. Since only about half the activity is lost either all the active sites are modified, decreasing the catalytic activity by half or only half the active sites are modified yielding half the number of inactive sites and an equal number of completely active sites.

An observation of some importance in elucidating the nature of the reaction is whether or not it is reversible. We have confirmed that neither 0.1 M ascorbic acid nor 0.1 M cysteine can reverse the inhibition. On the other hand, mercaptoethanol and mercaptoethylamine can reverse the inhibition induced by iodine. This obsernation alone argues strongly against an iodination reaction, which would not be reversed so easily or so rapidly. It is remarkable that β-mercaptoethylamine can reverse the inhibition while cysteine; which is α-carboxyl β-mercaptoethylamine, cannot do so. The presence of the carboxylate group is the only difference and its effect on the mercaptan reactivity is rather small. We are obliged to conclude that the presence of the carboxylate anion actively prevents the reactivation reaction. An explanation of this may be the presence close to or in the active centre of another carboxylate ion; mutual electrostatic repulsion would keep the reagent at a distance.

The effect of pH on the rate of the reactivation reaction is also unusual. The solid line in Fig. 3 is drawn from Eqn. 5.

$$k_2' = \frac{\bar{k}_2\left(1 + \frac{H}{K_1}\right)}{\left(1 + \frac{H}{K_2}\right)} \qquad (5)$$

The numerical values used were $pK_1 = 6.20$, $pK_2 = 8.00$, and $\bar{k}_2 = 5.91\ M^{-1} \cdot sec^{-1}$. $\bar{k}_2$ is the pH-independent second-order rate constant.

The physical interpretation of this equation is that the rate of the reactivation process is proportional to the concentration of the base form of an acid with a pK_a of 8.00 (pK_2), and proportional to the concentration of the protonated form of an acid with a pK_a of 6.20 (pK_1). At pH values well below pH 6.20 the enhancement due to the presence of the protonated acid (K_1), is negated by the protonation of the group (K_2) required in the base form. We suggest that the pK_a of 6.2 is contributed by an histidine residue; the residue which has a pK_a of 8.0 is more difficult to identify, and may be a lysine, cysteine or tyrosine. However, it is not the mercaptan group of the mercaptoethanol since this has a pK_a of 9.43 (ref. 18). There is no evidence that either of these two groups actually react with the iodine in the initial inactivation process. On the contrary, since the initial inactivation by iodine is not pH dependent[5] it is very unlikely that either of these two groups are directly involved. Their participation is more likely to be due to configurational alterations which are induced when they are deprotonated.

The kinetics of invertase and iodine–invertase revealed three interesting observations. Firstly, there is a significant decrease in the value of k_{+2} following reaction

with iodine. However, the pH dependence of k_{+2} is not substantially altered, although the present data do not exclude small shifts in the pK_a values of the groups involved.

From Figs. 4a and 4b it is seen that in fact at the higher pH values the observed k'_{+2} values are greater than predicted by Eqn. 3. This divergence was first observed by OTTOLENGHI[19] who has established this fact by very careful and extended kinetic measurements. The present observations are consistent with his conclusion that there is a second group with a pK_a of 8.65 which affects the enzyme reaction in the protonated form. We have fitted our data by using a value for the single pK_a of 6.9. We suggest that the group with the pK_a of 6.9 is an histidine residue. If this imidazolium group was iodinated its pK_a value would have dropped substantially. Since this was not observed we conclude that this histidine was not iodinated.

The decrease in k_{+2} in iodine–invertase may be due either to a decrease in the intrinsic k_{+2} of each catalytic site or to a decrease in the number of effective active sites. We favour the view that iodine treatment approximately halves the number of effective active sites. The lines in Figs. 4a and 4b are almost superimposable when the vertical ordinate is altered by a factor of two. There is a slight discrepancy which may be experimental error or may reflect a small additional effect of the iodine reaction.

We have noted that iodine may react with tyrosine, methionine or tryptophan residues. We have shown the apparent absence of iodotyrosine by spectral evidence. Therefore, it is very unlikely that the iodine has reacted with the tyrosine residues. Determination of the variation of k_{+2} with pH of nitrated invertase will enable one to decide whether tyrosine contributes to the k_{+2} at alkaline pH.

The alkylation of invertase by iodoacetamide but not by iodoacetic acid recalls the comparison between β-mercaptoethylamine and α-carboxyl β-mercaptoethylamine (cysteine) in the reactivation of iodine–invertase. Although iodoacetamide is more reactive than iodoacetic acid, one would reasonably expect that 0.1 M sodium iodoacetate at pH 9.0, 25° would alkylate all available nucleophiles in 325 min. Consequently, we again draw the conclusion that the acid is non-reactive because of the presence of its carboxylate anion. Again, we assume from this the presence of a carboxylate anion at or very near the active site.

However, it is also clear that the iodoacetamide does not react with the same atom as does the iodine, since the iodine reaction is reported[5] to be independent of pH, but Fig. 7 shows clearly that the iodoacetamide reaction is pH dependent. Since the reagent is not an acid itself, the acid group must reside in the enzyme. Our data are seen to fit Eqn. 4 reasonably when a pK_a value of 7.0 is used (Fig. 7). Again, we presume this to be a histidine residue; but this histidine is not being alkylated. If it were, then the equation would be of the form

$$k_2' = k_2/\left(1 + \frac{H}{K_a}\right) \tag{6}$$

The physical interpretation we put upon Eqn. 4 (Fig. 7) is that an acid group with a pK_a well in the alkaline region is being alkylated; and that at neutral pH values a histidine residue is maintaining a higher rate of reaction than would be expected for a single isolated ionizing species. The molecular explanation for this rate enhancement is not apparent, but the observation is that an acid group can show rates of reaction which are independent of pH over restricted ranges. Such a pheno-

menon has been observed previously for yeast[20] and for liver[21] alcohol dehydrogenase.

The second-order rate constant for the reaction between iodoacetamide and invertase at pH 7.8 was estimated to be $1.48 \cdot 10^{-4}$ $M^{-1} \cdot sec^{-1}$. The rate constant for the reaction between mercaptide ion and iodoacetamide[20] is about $5 \cdot 10^6$ larger than this value. Assuming a pK_a of about 10 for the reactive group, the invertase reaction would still be about 10 000 times less reactive. Consequently, it is unlikely that this is a reaction with cysteine; it is probably reacting with a lysine residue.

The reactions of invertase with iodine or with iodoacetamide appear to be independent since prior reaction with one reagent does not affect reaction with the other. Presumably, the reagents attack two separate functional groups at the active site, since they both lead to some inhibition.

The results with CNBr establish that this reagent is able to inhibit external yeast invertase by two different reactions; a very fast and a slow reaction. Since the slow reaction is independent of pH and occurs in mildly acid medium it is very likely that this is a reaction between a methionine sulphur atom and CNBr. We can conclude that reaction with a methionine leads to enzyme inhibition, although, of course, we are unable from this to ascertain the relationship of this methionine to the active site. Since it is likely that iodine also reacts with a methionine residue the question arises whether the two reactions are independent and it was found that they were. Iodine–invertase was inhibited by CNBr in the same way as native invertase. The iodine reaction does not prevent the reaction of CNBr. We must conclude, again, that these two reactions occur at different atoms.

The extent of the rapid reaction with CNBr is dependent on the pH. This may be explained by a relatively slow pH-dependent conformational equilibrium in which only one of the forms reacts rapidly with CNBr.

We observed that methyl mercury nitrate inhibited invertase, but that 50% inhibition occurred at $1.2 \cdot 10^{-6}$ M inhibitor. We conclude that the inhibitor is probably forming a chelate involving the nitrogen atom of the presumptive active site imidazole and a sulphur atom of another residue, probably a methionine.

In summary, we observed that the iodine inactivation of invertase was reversible by mercaptoethanol and mercaptoethanolamine but not by cysteine.

Iodoacetamide, but not iodoacetic acid, is able to alkylate and inhibit invertase. We conclude that there is a carboxylate anion at or near the active site which keeps anionic reagents at a distance.

The nature of the iodine reaction is deduced from the data presented in this paper. Since the inhibition is reversed by mercaptoethanol it is probably an oxidation reaction and not an iodination. This conclusion is further supported since it was found that the active site imidazole was not iodinated, there was no spectral evidence of iodotyrosine and nitration of tyrosines was not inhibitory. The evidence suggests that iodine is oxidising either a methionine residue or a cysteine residue. The reaction is independent of pH (ref. 5) and there is no cysteine detectable in internal yeast invertase[22] which nonetheless can be inactivated and reactivated in the same way as external invertase[11,12]; from this, we conclude that iodine is oxidising a particularly reactive methionine sulphur atom. Reaction of invertase with CNBr reveals that reaction with methionine in invertase does lead to enzyme inhibition.

Finally, on the basis of the foregoing conclusions we propose that yeast invertase has the following mechanism of action, depicted diagrammatically in Fig. 13.

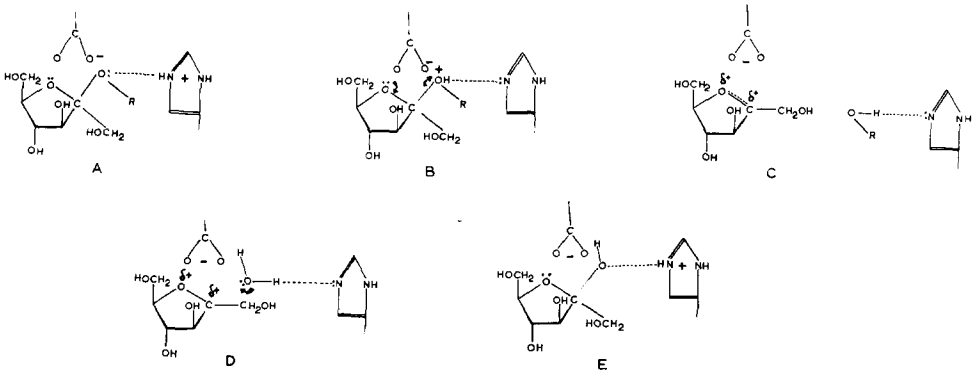


Fig. 13. Proposed mechanism of action of yeast invertase (β-D-fructofuranosidase).

The active site includes an histidine residue with a pK_a of 6.8–6.9 and a carboxylate anion. The first step, of course, is binding of the substrate to an adjacent substrate site which is stereospecific for β-D-fructofuranosides. This is followed by transfer of a proton from the active site imidazolium cation to the glycosidic oxygen. The positive charge on the glycosidic oxygen will tend to draw the electrons from the furanose ring oxygen and from C-2. Then there will be departure of the alcohol leaving behind a somewhat unstable intermediate carbonium ion in which the electron deficiency will spread over both C-2 and the ring oxygen. The active site carboxylate anion will function during this and the previous stage by stabilizing the electron-deficient species. The next stage is the attack of the C-2 cation by a nucleophilic oxygen atom of an alcohol or water to yield a fructoside or fructose. The final stage is dissociation of the product. The stereochemistry of the reaction need not be defined by the presence of the intermediate carbonium ion. It is erroneous to argue that retention of optical activity must mean that the reaction pathway includes one or two Sn2 reactions. Since the reacting species is the enzyme–substrate complex it is apparent that the protein may impose geometric restrictions on possible reactions so that a carbonium ion adsorbed on to the enzyme surface may be attacked from one side only to yield an optically active product. Accordingly, the presence of β-D-fructose as a product does not argue against the intermediate carbonium ion.

ACKNOWLEDGMENT

This work was supported by the Science Research Council and by the Distillers Company Limited.

REFERENCES

1 H. VON EULER AND S. LANDERGREN, *Biochem. Z.*, 131 (1922) 386.
2 H. VON EULER AND K. JOSEPHSON, *Z. Physiol. Chem.*, 127 (1923) 99.
3 K. MYRBÄCK, *Z. Physiol. Chem.*, 158 (1926) 160.
4 K. MYRBÄCK AND E. WILLSTAEDT, *Arkiv Kemi*, 12 (1958) 203.
5 K. MYRBÄCK, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 4, Academic Press, New York, 2nd ed., 1960, p. 389.
6 S. SHALL AND A. WAHEED, *Biochem. J.*, 111 (1968) 33P.

7 A. WAHEED AND S. SHALL, *Abstr. 4th Meeting Federation European Biochem. Soc.*, Oslo, No. 481 (1967).
8 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
9 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
10 W. W. CLELAND, *Nature*, 198 (1963) 463.
11 A. BASEER, PH. D. THESIS, University of Sussex, 1970.
12 A. BASEER AND S. SHALL, in preparation.
13 M. SOKOLOVSKY, J. F. RIORDAN AND B. L. VALLEE, *Biochemistry*, 5 (1966) 3582.
14 J. F. RIORDAN, M. SOKOLOVSKY AND B. L. VALLEE, *Biochemistry*, 6 (1967) 358.
15 D. W. HOUGH AND S. SHALL, *FEBS Letters 8* (1970) 243.
16 S. G. WALEY, *Biochim. Biophys. Acta*, 10 (1953) 27.
17 J. SCHREIBER AND B. WITKOP, *J. Am. Chem. Soc.*, 86 (1964) 2441.
18 A. ALBERT AND E. P. SERJEANT, *Ionization Constants of Acids and Bases*, Methuen, London, 1962, p. 135.
19 P. OTTOLENGHI, *European J. Biochem.*, 18 (1971) 544.
20 E. P. WHITEHEAD AND B. R. RABIN, *Biochem. J.*, 90 (1964) 532.
21 N. EVANS AND B. R. RABIN, *European J. Biochem.*, 4 (1968) 548.
22 S. GASCON, N. P. NEUMANN AND L. O. JAMPEN, *J. Biol. Chem.*, 243 (1968) 1573.
23 H. FRAENKEL-CONRAT, in P. D. BOYER, H. LARDY AND MYRBÄCK, *The Enzymes*, Vol. 1, Academic Press, New York, 2nd ed., 1959, p. 603.
24 M. E. KOSHLAND, F. M. ENGELBERGER, M. J. ERWIN AND S. M. GADDONE, *J. Biol. Chem.*, 238 (1963) 1343.
25 N. P. NEUMANN AND J. O. LAMPEN, *Biochemistry*, 6 (1967) 468.
26 J. FRIIS AND P. OTTOLENGHI, *Compt. Rend. Trav. Lab. Carlsberg*, 31 (1959) 259.
27 S. GASCON AND P. OTTOLENGHI, *Compt. Rend. Trav. Lab. Carlsberg*, 36 (1967) 85.
28 D. L. MORRIS, *Science*, 107 (1948) 254.
29 C. W. CHUNG AND W. J. NICKERSON, *J. Biol. Chem.*, 208 (1954) 395.