Studies on Mutarotase: Photooxidation Reactions and Nature of the Enzyme Catalysis†

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ABSTRACT: The nature of the anomerization of sugars catalyzed by mutarotase (aldose 1-epimerase) has been investigated. The purified enzyme is rapidly photoinactivated in the presence of Methylene Blue or Rose Bengal as sensitizing dyes. The rate is first order to less than 2% residual activity and is markedly and specifically reduced by substrate sugars. A constant photoinactivation rate was observed in the range pH 5.5–8.0 which increased rapidly at higher values. pH–photooxidation profiles of certain amino acids and their derivatives were measured and paralleled the theoretical ionization curves with half-maximal rates at the following pH values: L-histidine, 6.0; imidazole, 7.0; L-cysteine, 8.2; *N*-acetyl-L-tyrosine ethyl ester, 9.6; tyrosine, 10.1. Whereas the rate for L-methionine was relatively constant between pH 4 and 10, that for L-tryptophan showed a rapid increase between pH 8 and 10 similar to that observed for mutarotase. The relative kinetic constants for photooxidation of the enzyme by Rose Bengal *vs.* Methylene Blue were the same as those for tryptophan. Photoinactivation is also accompanied by stoichiometric loss of tryptophan from the enzyme. Mutarotase reduces the activation energy of the spontaneous mutarotation of glucose by 11.6 kcal, a figure which is similar to that for distortion from the chair to the half-chair or pseudoacyclic form of the sugar ring. In addition, interaction with substrate sugars is accompanied by a substantial conformational change in the enzyme as detected by membrane filtration analysis. It is concluded that potent nucleophiles such as histidine are probably not present or required in the active center of mutarotase. It is suggested that tryptophan is involved as a glucose-binding site and that catalysis of the mutarotation reaction is accomplished by a ring-distortion type of mechanism.

The mutarotation of sugars in solution is an example of a general acid–base-catalyzed reaction. It is generally believed that the open-chain or aldehydo form of the sugar is an intermediate in the process (Pederson, 1934; Cantor and Peniston, 1940; Swain and Brown, 1952).

The enzyme mutarotase or aldose 1-epimerase (EC 5.5.33) also is a catalyst of the interconversion of the anomeric forms of glucose and related sugars. Whereas the hydroxyl ion, which is one of the best inorganic catalysts of the mutarotation reaction, has a catalytic coefficient of about 10^3 min^{-1}, that of the active center of bovine kidney mutarotase is 1.0×10^6 or some three orders of magnitude greater (Bailey *et al.*, 1969).

The nature of the enzyme catalysis was studied by Bentley and Bhate (1960) by use of D-[$^{18}O_1$]glucose. The enzymatic reaction was shown not to involve dehydration, dehydrogenation or single displacement reactions.

In view of the observed effectiveness of histidine as a catalyst of mutarotation (Westheimer, 1937), a role for this amino acid in the active site has seemed possible. Wallenfels *et al.* (1965) found that the enzyme activity is dependent upon the presence of free SH groups, and based on the pH optimum, suggested that the active center may contain both a histidine residue and two SH groups so that the catalysis of ring opening and closing may be accomplished by sequential protonation–deprotonation reactions. Bentley and Bhate (1960), however, by measurement of the relative mutarotation rates in H_2O and D_2O have shown that the catalysis of mutarotation by histidine and histidyl histidine differs significantly from that of the enzyme.

Because of the sensitivity of histidine in both the free and the protein-bound form to photooxidation (Westhead, 1965) the studies reported here on the photoinactivation of a purified bovine kidney mutarotase were undertaken.

Materials and Methods

Mutarotase was isolated in pure form from bovine kidney cortex by a modification of the previously reported procedure (Bailey *et al.*, 1969). Full details will be given elsewhere (Bailey *et al.*, 1973).

Measurements of mutarotase activity were made by the polarimetric procedure described previously (Bailey *et al.*, 1967). Photochemical oxidations of the purified bovine kidney mutarotase using Methylene Blue or Rose Bengal as sensitizing dyes were carried out by a modification of the procedure of Westhead (1965). Reactions were carried out in a thermostated glass reaction vessel using a 750-W slide projector as light source. Photoinactivation rates of certain amino acids and their derivatives were determined by the rate of oxygen uptake measured with an oxygen electrode. The tryptophan content of the native and the photooxidized enzyme was measured by reaction with *N*-bromosuccinimide according to the procedure of Spande and Witkop (1967). Conformational changes induced in the enzyme by substrate sugars were monitored in a Diaflo pressure cell using an XM50 membrane. Purified mutarotase (6000 units, 250 μg) in Krebs–Ringer bicarbonate buffer (pH 7.4) was placed in the cell under pressure. Fractions of 2 ml each were collected and enzyme content was assayed. Test compounds were then added to the cell and changes in the filtration rate were monitored as described in the footnote to Table III.

Results

Photochemical Oxidation Studies. Mutarotase was rapidly inactivated when exposed to visible light in the presence of

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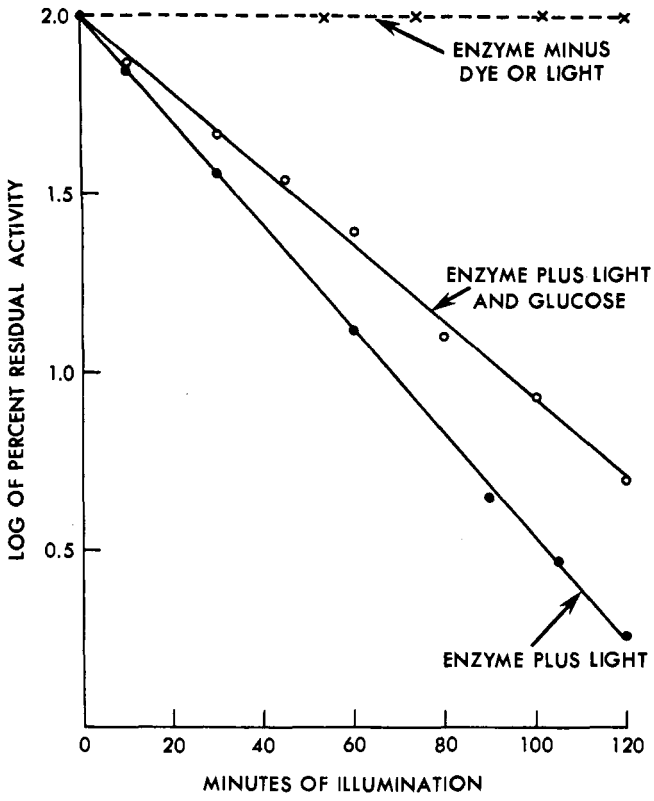

FIGURE 1: Photochemical inactivation of bovine kidney mutarotase. Solutions containing 560 units (21.8 μg) of pure bovine kidney mutarotase in 10 mM Tris-HCl buffer (pH 7.0) were incubated at 12.5° in a thermostated glass reaction vessel. The light source was a 750-W projector and the solutions were stirred with a small magnetic stirrer. One sample contained 0.02% Methylene Blue and was illuminated; a second illuminated sample contained 200 mM D-glucose and 0.02% Methylene Blue; no addition was made to a third illuminated sample. The final sample containing 0.02% Methylene Blue was kept in the dark. At the indicated times, 0.1-ml aliquots were removed to foil-covered tubes containing 11.0 ml of buffer (5 mM EDTA, pH 7.2) and residual enzyme activity was determined by the standard polarimetric assay procedure. There was no loss of activity in the absence of Methylene Blue or light. The rate of the photochemical inactivation was first order down to less than 2% residual activity with a rate constant of 0.146 min^{-1}. This rate constant was reduced to 0.104 min^{-1} in the presence of the substrate glucose.

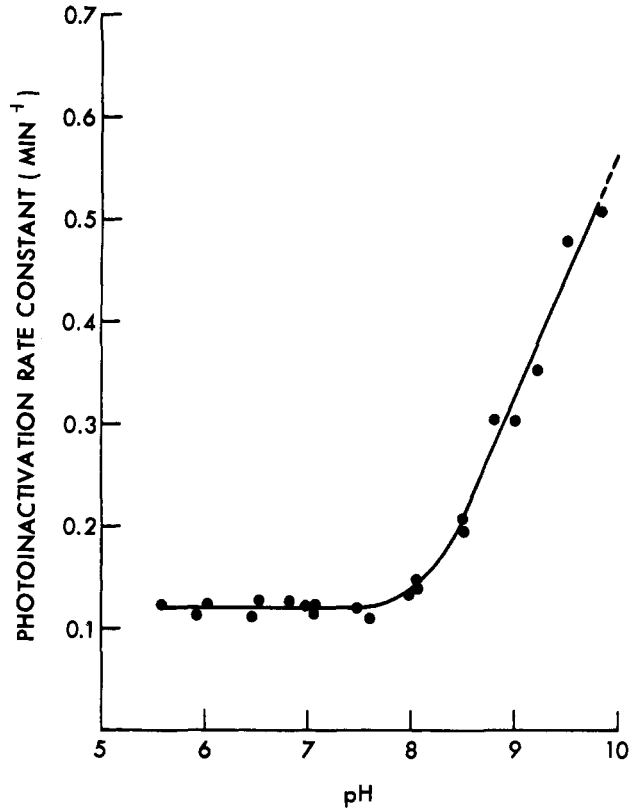

FIGURE 2: Influence of pH on photoinactivation of mutarotase. Solutions of the pure enzyme (11.2 μg, 300 units) were photooxidized using 10 μg/ml of Rose Bengal as sensitizing dye in solutions of various pH values as described in Methods. The first-order rate constants for the inactivation were obtained by plotting the logarithm of the residual activity against time. At the higher pH values the rate constants were corrected for the rate of inactivation in the absence of dye due to alkaline denaturation.

Methylene Blue (Figure 1). The rate was first order and linear down to less than 2% residual activity and was markedly reduced by the presence of substrate. There was no loss of activity in the presence of Methylene Blue if the enzyme was kept in the dark. A similar monophasic rate of inactivation was also obtained with Rose Bengal as the sensitizing dye. A constant low rate was observed between pH 5.6 and 7.6. At higher pH values the rate increased rapidly (Figure 2). Above pH 10 the alkaline denaturation rate of the enzyme became too rapid to enable accurate rate determinations to be made. At neutral pH D-glucose decreased the rate of inactivation but at higher pH the sugar itself was photochemically oxidized. α-Methyl D-glucoside, a competitive inhibitor of the enzyme which is stable under the same conditions, was therefore used at the higher pH values (Figure 3). This compound also reduced the rate of photoinactivation from 0.52 to 0.18 min^{-1}. The effect was specific since α-methyl D-mannoside, which is not an inhibitor of mutarotase, had no effect on the photoinactivation reaction (Table I). The noncompetitive inhibitor Hg^{2+} increased the photosensitivity of the enzyme. Other competitive inhibitors, L-fucose and L-xylose, protected the enzyme from photoinactivation and the effect was concentration dependent (Table I).

Photochemical oxidation of the enzyme was compared with that of certain amino acids and their derivatives under the same experimental conditions. The amino acids histidine, tryptophan, methionine, and cysteine were photosensitive in varying degree. The influence of pH on the photooxidation of these compounds is shown in Figure 4. Methionine was unaffected by pH changes and tryptophan was moderately affected. Histidine, imidazole, tyrosine, and cysteine were strongly influenced by pH changes. There appears to be excellent agreement between the relative rates of oxidation of these latter compounds and their theoretical ionization curves. Hg^{2+} completely inhibited the oxidation of cysteine over a broad pH range. However, the sugar inhibitors had no effect on the photochemical oxidation of the various amino acids.

The reaction was also strongly influenced by the concentration of the amino acid. A reciprocal plot of the velocity against amino acid concentration produces a straight line indicating Michaelis–Menten saturation-type kinetics for the photosensitizing dye. Thus each amino acid has a characteristic affinity constant for the dye-sensitized photooxidation reaction. The comparative values for the half-saturation concentrations, K_s, and the maximum velocities for Rose Bengal *vs.* Methylene Blue are shown in Table II. The relative sensitivity of the enzyme to the two dyes (1.48:1) was most closely matched by the amino acid tryptophan (1.43:1).

In a separate experiment a sample of purified mutarotase

TABLE I: Influence of Sugars and Inhibitors on the Photooxidation Rate of Purified Mutarotase.

Compound Added	K_m or K_i (mM)	Concn (mM)	pH	Rate Constant for Photoinactivation (min^{-1})		% Inhibn
				Control	Plus Inhibitor	
α-Methyl D-glucoside	9.6	200	9.5	0.518	0.178	66
α-Methyl D-glucoside	9.6	188	7.0	0.217	0.097	55
α-Methyl D-glucoside	9.6	20	7.0	0.217	0.148	32
L-Fucose	2.0	2.5	7.0	0.217	0.166	23
L-Fucose	2.0	45.7	7.0	0.217	0.087	60
L-Xylose	2.8	4	7.0	0.217	0.167	23
L-Xylose	2.8	45.7	7.0	0.217	0.084	61
α-Methyl D-mannoside	150	200	9.5	0.518	0.501	3
Control (dark)			9.5	0.000		
Hg^{2+} (dark)		0.005	9.5	0.220		
Hg^{2+} (light)		0.005	9.5	0.518	0.791	-10^a

[a] Corrected for dark reaction. Pure bovine kidney mutarotase (300 units; 11.3 μg) was illuminated in a thermostated glass reaction vessel at 12.5° using a 750-W projection lamp. The solutions were buffered either at pH 7.0 with 10 mM Tris-HCl or at pH 9.5 with 20 mM glycine–NaOH and the substrate and inhibitor sugars were added as indicated. At zero time a sample of Rose Bengal was added to the stirred solution to give a final concentration of 10 μg/ml. Samples of the solution were withdrawn at intervals to foil-covered tubes containing EDTA buffer (5 mM, pH 7.2) for measurement of mutarotase activity as described previously.

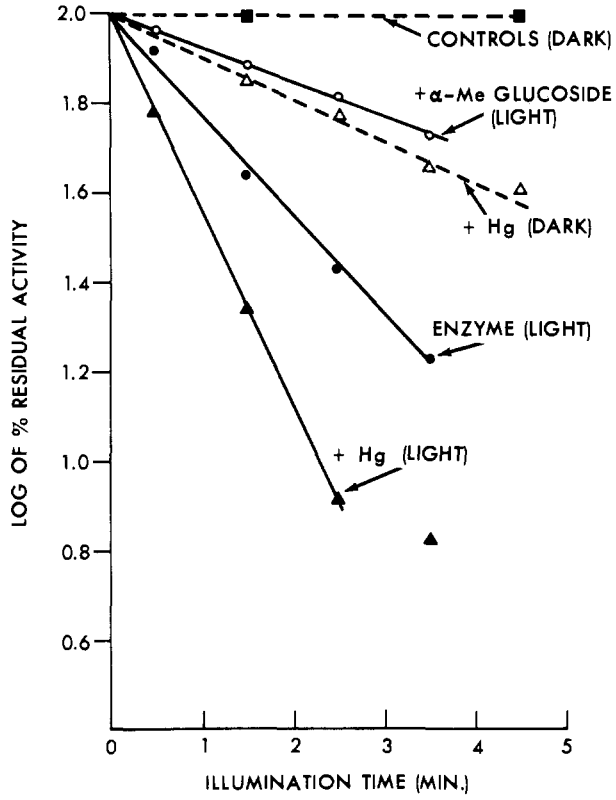

FIGURE 3: Influence of inhibitors on photoinactivation of mutarotase at pH 9.5. Solutions of pure bovine kidney mutarotase (11.2 μg, 300 units) in sodium glycinate buffer (20 mM, pH 9.5) were incubated at 25° in a thermostated all-glass reaction vessel. Where indicated α-methyl D-glucoside (200 mM) or $HgCl_2$ (5 mM) was added. The solutions were illuminated from a fixed distance (10 cm) using a 750-W projector. At zero time 10 μg/ml of Rose Bengal was added and samples (0.1 ml) were withdrawn at intervals to foil-covered tubes containing EDTA buffer (5 mM, pH 7.2) for subsequent assay of mutarotase remaining using a polarimetric assay procedure. L-Cysteine (5 mM) was added to the assay buffer when the samples contained Hg^{2+}.

(19,800 units; 750 μg) dissolved in 3.5 ml of Tris buffer (10 mM, pH 7.4) was divided into two portions. One portion was photooxidized in the presence of Methylene Blue to 62% inactivation. The tryptophan content of the photooxidized and

TABLE II: Relative Kinetic Constants for Photooxidation of Amino Acids and Mutarotase Using Methylene Blue and Rose Bengal.[a]

Test Compound	Kinetic Parameters for Photooxidation				
	Rose Bengal (RB)		Methylene Blue (MB)		
	K_s (mM)	V_{max}	K_s (mM)	V_{max}	RB:MB
Histidine	4.4	1.34	3.44	0.233	5.75
Methionine	11.3	1.01	20.0	0.325	3.08
Cysteine	2.7	0.50			
Tryptophan	0.36	0.050	1.78	0.035	1.43
Tyrosine	2.0	0.010	16.6	0.022	0.45
Mutarotase		0.217		0.146	1.48

[a] The kinetic constants for the photochemical oxidation of the amino acids at pH 7.0 and 10° with Rose Bengal as the photosensitizing dye were obtained from a reciprocal plot of reaction rate *vs.* amino acid concentration, where K_s is the amino acid concentration at which the rate is half-maximal and V_{max} is in units of micromoles of amino acid oxidized per minute based upon an observed equivalence of 1 mol of oxygen for histidine and methionine, 2 mol for tryptophan and tyrosine, and 0.5 mol for cysteine. The values for Methylene Blue were recalculated from the data of Weil (1965). Photoinactivation of mutarotase at pH 7.0 was measured in separate experiments as described in the legends to Table I and Figure 1. The relative photoinactivation rates for the enzyme with Rose Bengal *vs.* Methylene Blue were essentially the same as that obtained for tryptophan.

TABLE III: Detection of Substrate-Induced Conformational Changes in Mutarotase by Membrane-Filtration Analysis.[a]

Test Compound	XM50 Membrane	Filtration Fraction: Alone	Plus Test Sugar	Plus Test Sugar Plus Glucose
Substrates				
D-Glucose	1	0.35	0.21	
D-Glucose	2	0.50	0.32	
D-Glucose	3	0.47	0.33	
D-Galactose	2	0.58	0.28	
D-Xylose	1	0.42	0.21	
Nonsubstrates				
L-Glucose	1	0.34	0.33	
D-Mannose	3	0.48	0.45	
2-Deoxy-D-glucose	2	0.56	0.50	0.32
Sucrose	2	0.58	0.60	0.44

[a] Samples of purified mutarotase (6000 units, 250 μg) in 40 ml of Krebs–Ringer bicarbonate buffer (155 mM, pH 7.4) were filtered from a 50-ml capacity Diaflo pressure cell equipped with magnetic stirrer. Different samples of XM50 membrane having a molecular weight cutoff of approximately 50,000 were used as indicated. The cell was pressured with 5% CO_2 in N_2 (15 psi) and flow rate was 1.5 ml min^{-1}. Five samples of 2 ml each were collected. The test sugar was then added to the filtration cell in a final concentration of 0.3% and sample collection was continued. Aliquot portions of both the filtrate and the solution remaining in the filtration cell were removed at intervals and analyzed for mutarotase content using the polarimetric assay procedure. The filtration fraction in the table above was calculated as the ratio of the concentration of enzyme in the filtrate to that in the filtration cell after making appropriate corrections for volume changes in the cell between the sampling intervals. The procedure was repeated for the various test sugars shown above. In some experiments (2-deoxyglucose, sucrose) glucose (0.3%) was added and further fractions were collected. Significant decreases in the filtration fraction of 30–50% were found for all substrates tested, whereas nonsubstrate sugars produced only small or negligible effects on the filtration characteristics of the enzyme.

control samples was measured by oxidation with *N*-bromosuccinimide by the procedure of Spande and Witkop (1967) in 8 M urea. A value of 3.23 mol of tryptophan/mol was obtained for the control and only 0.96 mol/mol corresponding to 70% destruction of tryptophan residues for the photoinactivated sample.

Conformational Changes in the Enzyme. Filtration of the purified enzyme through a membrane of graded pore size (Diaflo XM50) was decreased in the presence of substrate sugars. The filtration fraction (*i.e.*, the concentration of enzyme in the filtered solution compared to that in the pressure cell) averaged 0.44 and ranged from 0.35 to 0.50 with different samples of XM50 membrane. Addition of sugars which are substrates of mutarotase (D-glucose, D-galactose, D-xylose) produced a 38% average decrease in the filtration fraction (Table III) whereas no significant change was induced when nonsubstrate sugars (L-glucose, D-mannose, 2-deoxy-D-glucose, sucrose) were added to the pressure cell.

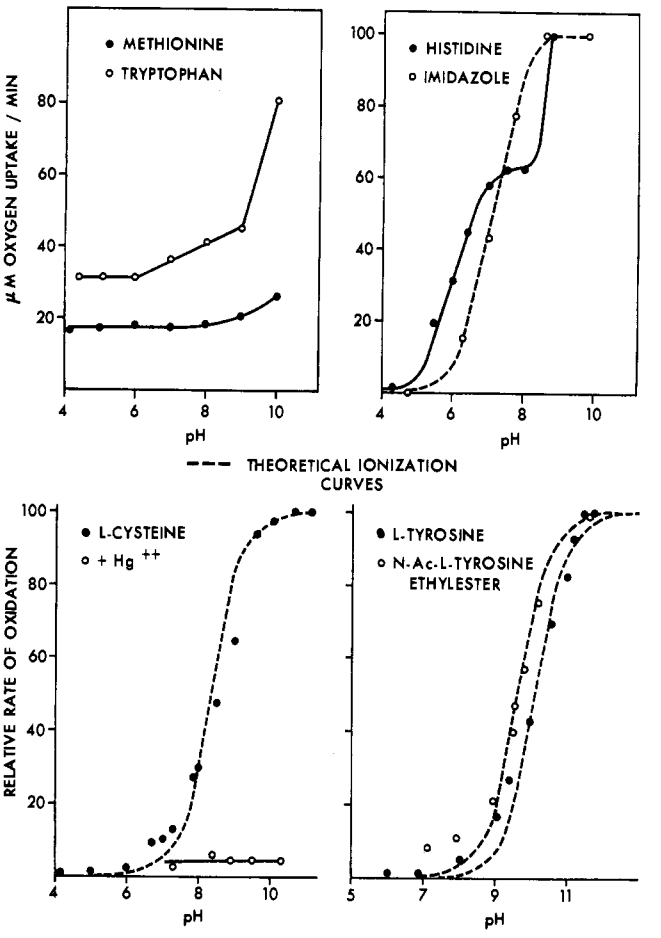

FIGURE 4: Influence of pH on photooxidation of amino acids and derivatives. Solutions of the amino acids (200 μM) were photooxidized at 25° using 10 μg/ml of Rose Bengal in 50 mM phosphate or Tris buffers of varying pH as described in Methods. The dotted curves are the theoretical ionization curves and indicate that the nonprotonated amino acids are the photosensitive forms.

Discussion

The influence of substrates and competitive inhibitors on photoinactivation of mutarotase suggests that the loss of activity is connected with photodynamic events at or near the active site of the enzyme (Table I). All of the competitive inhibitors reduce the rate of inactivation to the same extent at concentrations near their K_I's and also near saturation. As they did not affect the oxidation of individual amino acids their effect appears to be specific for the enzyme. In addition, a relationship of the pH dependence of photoinactivation to some group at the active site is suggested by the observation that α-methyl glucoside, a competitive inhibitor of mutarotase, reduced the photoinactivation rate of the enzyme both at pH 7 and 9.5 (Table I). This indicates a strong pH dependence of photochemical inactivation with and without a protecting group at the active site of the enzyme. It would thus seem that a similar group is being photochemically oxidized under both conditions. Since histidine is a good catalyst of the mutarotation of sugars (Westheimer, 1937), Hucho and Wallenfels (1971) have argued on the basis of kinetic data and pH profiles that histidine is part of the active site of mutarotase from *Escherichia coli*.

Bentley and Bhate (1960) found, however, that the k_H/k_D ratio for the enzyme-catalyzed reaction was similar to that observed for the spontaneous mutarotation reaction in H_2O and

D_2O, and that the k_H/k_D ratio of 3.68 for histidine-catalyzed mutarotation was much higher than that observed for mutarotase (1.78) or other acid catalysts. The possibility that a lower ratio might be found for a histidine present in peptide linkage was shown to be unlikely since there was no significant change in the k_H/k_D ratio when histidylhistidine was substituted for histidine (3.85 *vs.* 3.68).

The pH-dependent photooxidation of proteins containing histidine residues in the active center usually follows that observed for imidazole (Figure 4), a finding which is well documented for enolase (Westhead, 1965) and bacterial collagenases (Takahashi and Seifter, 1969). The pH profile for photoinactivation of mutarotase (Figure 2) bears no resemblance to that for either histidine or imidazole. It is difficult to envisage a possible environment for histidine residue which would shift the pK by more than 3 units. If histidine is present in the active site of bovine kidney mutarotase it would have an unusual pK which is quite incompatible with the pH–activity profile observed for the enzyme-catalyzed mutarotation of sugars.

Both cysteine and tyrosine are photochemically oxidized in their ionized state (Figure 4). However, these amino acids react at negligible rates in acid media, whereas mutarotase is inactivated at an appreciable rate which is constant between pH 5.6 and 7.6. In addition mercuric ions greatly reduce the rate of photochemical oxidation of cysteine (Figure 4) but increase the rate of photoinactivation of mutarotase (Figure 4 and Table I). Finally, acetylation studies on the tyrosine residues of the enzyme indicate tyrosine is not involved in the active site (Fishman *et al.*, 1973).

Examination of the pH-dependent photochemical oxidations of model amino acids (Figure 4) indicates that tryptophan is sensitive to pH changes on the alkaline side, an observation also made by Sluyterman (1962) and Weil (1965). In addition, tryptophan is photooxidized at an appreciable rate in acid media. Amino acid analysis indicates that mutarotase contains five tryptophan residues per mole (Fishman *et al.*, 1973).

A function for tryptophan in substrate binding has been proposed for several enzymes. Crystallographic and protein modification studies indicated that tryptophanyl residues are part of the binding site of lysozyme (Chipman and Sharon, 1969). Oxidation of α-amylase by *N*-bromosuccinimide implicated one tryptophan residue in the active site of that enzyme and tryptophan also appears to be involved in the binding of biotin to avidin (Spande and Witkop, 1967).

One of the best methods of evaluating the role of tryptophan in the binding sites of proteins is by selective oxidation with *N*-bromosuccinimide. The tryptophan residues of mutarotase are readily oxidized by *N*-bromosuccinimide with concurrent inactivation of the enzyme, and a stoichiometric loss of tryptophan during photoinactivation is also found.

Bentley (1972) has discussed the influence of ring conformation and instability factors on the rate of mutarotation of sugars. The analysis indicates that the higher the degree of instability, the faster the spontaneous mutarotation. A sugar in the pyranose form is most stable when all of the hydroxyl groups are equatorial. All known substrates for mutarotase exist in the $^4C_1$ chair form and are thus relatively stable. If the enzyme, upon binding the substrate, promoted instability by distorting the ring, the mutarotational rate would be enhanced.

Recent studies on lysozyme indicate that, upon binding of substrate the pyranose ring of the sugar residue at the active site is distorted from its normal chair form to a half-chair form (Chipman and Sharon, 1969). It is believed that the distortion involves a free-energy change of 10 kcal. The activation energy of the reaction catalyzed by mutarotase was measured and found to be only 11 kcal/mol, *i.e.*, about 11.6 kcal/mol less than the value for spontaneous mutarotation (Bailey *et al.*, 1969).

Exchange studies indicate that the carbonium ion intermediate which would result from cleavage of the anomeric hydroxyl is not involved in spontaneous or enzymatic mutarotation (Bentley and Bhate, 1960). However, there appears to be no configurational restraint on the open-chain intermediate which could exist in a coplanar form bound to the enzyme. The change in filtration of mutarotase through molecular seive membranes in the presence of substrate may be related to the induction of conformational changes and ring strain in the substrate. Both L-glucose and 2-deoxy-D-glucose, which are competitive inhibitors of the enzyme-catalyzed mutarotation of glucose, can spontaneously mutarotate. Nevertheless, their interaction with the enzyme is not accompanied by an increase in their mutarotation rate, suggesting that in addition to binding to the active center a substrate must do so in a manner which induces the necessary conformational change in the enzyme protein. Phloridzin which is a potent inhibitor of glucose transport in kidney also induces a similar decrease in filtration of the enzyme when analyzed by the membrane filtration procedure (Kusiak and Bailey, 1972). Catalysis of mutarotation by increased ring strain due to conformational changes in the enzyme therefore is particularly appealing because the favorable changes in free energy would reduce the requirement for potent acids and nucleophiles in the active center and may explain why none are apparently present.

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