Role of Arginine Residue in Saccharopine Dehydrogenase (L-Lysine Forming) from Baker's Yeast†

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ABSTRACT: The baker's yeast saccharopine dehydrogenase (EC 1.5.1.7) was inactivated by 2,3-butanedione following pseudo-first-order reaction kinetics. The pseudo-first-order rate constant for inactivation was linearly related to the butanedione concentration, and a value of 7.5 M^{-1} min^{-1} was obtained for the second-order rate constant at pH 8.0 and 25 °C. Amino acid analysis of the inactivated enzyme revealed that arginine was the only amino acid residue affected. Although as many as eight arginine residues were lost on prolonged incubation with butanedione, only one residue appears to be essential for activity. The modification resulted in the change in V_{max}, but not in K_m, values for substrates. The inactivation by butanedione was substantially protected by L-leucine, a competitive analogue of substrate lysine, in the presence of reduced nicotinamide adenine dinucleotide (NADH) and α-ketoglutarate. Since leucine binds only to the enzyme·NADH·α-ketoglutarate complex, the result suggests that an arginine residue located near the binding site for the amino acid substrate is modified. Titration with leucine showed that the reaction of butanedione also took place with the enzyme·NADH·α-ketoglutarate·leucine complex more slowly than with the free enzyme. The binding study indicated that the inactivated enzyme still retained the capacity to bind leucine, although the affinity appeared to be somewhat decreased. From these results it is concluded that an arginine residue essential for activity is involved in the catalytic reaction rather than in the binding of the coenzyme and substrates.

Saccharopine dehydrogenase [N^{ϵ}-(L-glutaryl-2)-L-lysine: NAD oxidoreductase (L-lysine forming) EC 1.5.1.7] is the last enzyme in the α-aminoadipate pathway of lysine biosynthesis and catalyzes a reversible cleavage of saccharopine to L-lysine and α-ketoglutarate in the presence of a pyridine nucleotide coenzyme. The enzyme from baker's yeast is a monomeric protein containing 1 active site/mol of enzyme (Ogawa & Fujioka, 1978). Chemical modification studies in this laboratory have shown that the enzyme possesses one residue each of cysteine (Ogawa et al., 1979), histidine (Fujioka et al., 1980), and lysine (Ogawa & Fujioka, 1980) at the active site, all of which are essential for activity.

With the advent of the specific reagents such as phenylglyoxal (Takahashi, 1968) and butanedione (Yankeelov, 1970; Riordan, 1973) which modify arginine residues in proteins under mild conditions, a number of enzymes have been examined for the involvement of arginine residues in the catalytic mechanism. As a general function of arginine residue, its involvement as a cationic group in the binding of negatively charged substrates or coenzymes is postulated. Since the catalytic reaction of saccharopine dehydrogenase involves both negatively charged pyridine nucleotide and substrates, we have attempted to explore the involvement of arginine residues in the function of the enzyme by using 2,3-butanedione as an arginine modification reagent. The results described herein suggest that the enzyme is inactivated by the modification of an arginine residue located in the vicinity of the binding site for the amino acid substrate but the residue is not directly involved in the binding of either the coenzyme or substrates.

Experimental Procedures

Materials. NAD^+ and NADH (disodium salt) were obtained from Oriental Yeast Co., Tokyo, and 2,3-butanedione was from Wako Pure Chemicals, Osaka. 2,3-Butanedione was redistilled prior to use. L-[^{3}H]Leucine (60 Ci/mmol) was purchased from New England Nuclear. L-Saccharopine was prepared enzymatically as described previously (Fujioka & Nakatani, 1974). Saccharopine dehydrogenase was purified from baker's yeast (Oriental Yeast Co.) by the method of Ogawa & Fujioka (1978). The purified preparation was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and ultracentrifugation. The experiments described below were carried out with the homogeneous enzyme preparation. All other chemicals were of the purest grade available from commercial sources.

Reaction of Saccharopine Dehydrogenase with 2,3-Butanedione. The redistilled 2,3-butanedione was freshly diluted with 0.08 M Hepes[1] buffer before each experiment. Incubation of saccharopine dehydrogenase with butanedione was carried out in 0.08 M Hepes buffer (pH 8.0), containing 0.2 M KCl and 10 mM sodium borate at 25 °C, unless otherwise indicated. The extent of inactivation was determined by the measurement of residual enzyme activity on an aliquot removed from the incubation mixture. The saccharopine dehydrogenase activity was estimated by the rate of decrease of NADH at 340 nm in the direction of saccharopine synthesis in the assay mixture containing 0.1 mM NADH, 2.5 mM α-ketoglutarate, and 7.5 mM L-lysine in 0.1 M potassium phosphate buffer (pH 6.8).

Amino Acid Analysis. Saccharopine dehydrogenase was incubated with butanedione, and, at appropriate time, the reaction mixture was diluted by one-third with 6 N HCl to halt the reaction and to prevent regeneration of free arginine (Riordan, 1973). The acidified mixture was dialyzed overnight against 1 N HCl. The sample was then dried under vacuum and hydrolyzed in 6 N HCl in an evacuated sealed tube. Hydrolysis was at 110 °C for 22 h. Amino acid analysis was performed in an LKB 4400 amino acid analyzer. The control sample was treated similarly in the absence of butanedione.

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[1] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonate; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; NADPH, reduced NAD phosphate.

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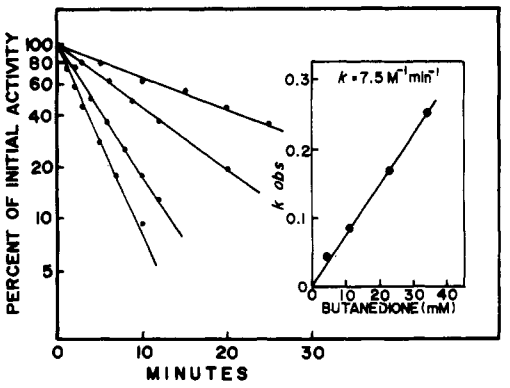

FIGURE 1: Time course of inactivation of saccharopine dehydrogenase by 2,3-butanedione. The enzyme (0.7 nmol) was incubated with butanedione in 0.1 mL of Hepes–KCl–borate buffer (pH 8.0). At times indicated, aliquots (2 μL) were removed for measurements of enzyme activity. The concentrations of butanedione from top to bottom: 4.6, 11.4, 22.8, and 34.2 mM. The activity corresponding to 100% was 161 μmol of NADH oxidized min^{-1} mg^{-1} of protein under the assay conditions described under Experimental Procedures. The inset shows the plot of apparent first-order rate constants obtained at various concentrations of butanedione against concentrations of the reagent.

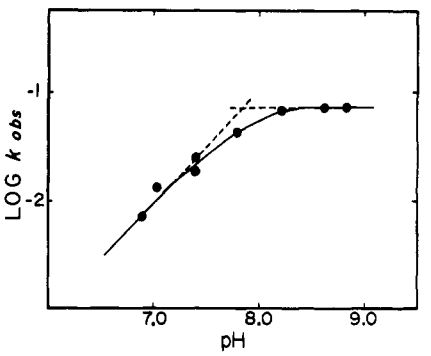

FIGURE 2: Effect of pH on the apparent first-order rate constant for inactivation. The enzyme (0.7 nmol) was incubated with 11.4 mM butanedione in 0.1 mL of 0.08 M Hepes buffer at the pH values indicated. The buffers contained 0.2 M KCl and 10 mM borate in addition. Values of the apparent first-order rate constant (k_{obsd}) were obtained from the pseudo-first-order kinetic plots.

Equilibrium Dialysis. The binding capacity of the butanedione-modified enzyme for L-leucine, a competitive inhibitor of L-lysine, was examined by the equilibrium dialysis method. The equilibrium dialysis was performed in a dialysis cell separated into two chambers by Visking 20/32 tubing pretreated with 5% Na_2CO_3–10 mM EDTA at 100 °C for 5 min. The inactivated enzyme that had been dialyzed against 0.1 M potassium phosphate buffer (pH 7.7), containing 1 mM EDTA (95 μM, 0.2 mL), was placed on one side of the membrane and an equal volume of the solution containing 1.6 mM NADH, 20 mM α-ketoglutarate, and L-[^{3}H]leucine (9.2 $\times$ 10^6 dpm/μmol) ranging from 0.2 to 3.0 mM in the same buffer was placed on the other side. The two solutions were allowed to equilibrate with gentle rocking for 16 h at 15 °C. During this period, the native enzyme lost no activity. Samples (30 μL) from each side were transferred to vials containing 0.97 mL of water and added with 9 mL of a scintillation fluid (toluene, 667 mL; Triton X-100, 333 mL; 2,5-diphenyloxazole, 5 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.4 g). The radioactivity measurements were made in an Aloka model LSC-903 liquid scintillation spectrometer. The amount of leucine bound by the enzyme was calculated from the difference in radioactivity between two sides of the dialysis cell and the specific radioactivity of [^{3}H]leucine.

Determination of Sulfhydryl Residues. The butanedione-inactivated enzyme was dialyzed overnight against 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The sulfhydryl content of the dialyzed enzyme was determined with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959) in 0.1 M Tris-HCl buffer (pH 8.0) in the presence of 0.2% sodium dodecyl sulfate and 0.45 M $(NH_4)_2SO_4$.

Protein Determination. The protein concentration was determined by the method of Lowry et al. (1951). A molecular weight of 39 000 for saccharopine dehydrogenase (Ogawa & Fujioka, 1978) was used in all calculations.

Results

Inactivation of Saccharopine Dehydrogenase by Butanedione. Incubation of saccharopine dehydrogenase with 2,3-butanedione in Hepes buffer containing KCl and sodium borate resulted in a progressive loss of enzyme activity. As shown in Figure 1, the inactivation followed pseudo-first-order reaction kinetics to <15% of the initial activity. The values of the apparent first-order rate constant were linearly related to the butanedione concentrations (Figure 1, inset), and the slope of the plot gave a value of 7.5 M^{-1} min^{-1} for the second-order rate constant.

Effect of Borate on Inactivation. In most of the enzymes studied thus far, the modification of arginine residues by butanedione is reported to be stimulated by borate ion and the reversal of inactivation to occur upon removal of the reagent. The addition of 10 mM borate to the reaction mixture of saccharopine dehydrogenase and butanedione at pH 8, however, had no effect on the rate of inactivation. Concentrations of borate higher than 20 mM caused a progressive decrease in the inactivation rate apparently due to the lowering of the effective concentration of the reagent through complex formation between butanedione and borate (Riordan, 1973). Although borate had no stimulatory effect on inactivation and the activity of the butanedione-treated enzyme was not appreciably recovered after extensive dialysis against buffers containing no borate, 10 mM borate was routinely included in the reaction mixtures to avoid possible reversal of inactivation.

Effect of pH on Inactivation. The effect of pH on the inactivation by butanedione was examined in Hepes buffer between pH 6.9 and 8.8. The rate of inactivation increased with increasing pH up to pH 8. The plot of apparent first-order rate constants obtained at different pH vs. pH gave a typical titration curve with a pK value of 7.8 (Figure 2).

Identification of Amino Acid Residue Modified by Butanedione. Table I shows the amino acid composition of the native and butanedione-inactivated enzymes. As seen from the table, arginine was the only amino acid residue affected by the butanedione treatment. Separate experiments showed that the fluorescence emission spectra (maximum at 340 nm; excitation wavelength, 280 nm) and ultraviolet absorption spectra of the native and butanedione-treated enzymes were identical (not shown), indicating the absence of appreciable reaction at tyrosine and tryptophan residues. The sulfhydryl content of the enzyme was not appreciably altered by the butanedione treatment; titration with 5,5′-dithiobis(2-nitrobenzoate) gave values of 2.85 and 2.45 cysteine residues/mol of the native and inactivated enzymes, respectively (Ogawa & Fujioka, 1978). A previous investigation has shown that saccharopine dehydrogenase possesses a cysteine residue which is highly reactive toward a variety of sulfhydryl reagents, the modification of which results in total loss of activity (Ogawa et al., 1979). With *p*-(chloromercuri)benzoate, it has been shown that a stoichiometric amount of the reagent causes complete inactivation instantaneously, and the activity is re-

Table I: Amino Acid Composition of Native and Butanedione-Treated Enzymes[a]

amino acid	native (mol/mol of enzyme)	butanedione-treated[b] (mol/mol of enzyme)
aspartic acid[c]	38.4	38.6
threonine	23.2	23.3
serine	17.2	17.6
glutamic acid[c]	27.6	28.0
proline	31.5	31.7
glycine	20.2	20.5
alanine	34.5	35.3
valine	20.8	21.1
methionine	2.5	2.5
isoleucine	19.4	19.3
leucine	32.0	32.0
tyrosine	7.9	7.8
phenylalanine	15.4	15.5
histidine	10.5	10.9
lysine	24.6	24.9
arginine	19.4	11.4

[a] Samples for amino acid analysis were prepared as described under Experimental Procedures. The number of residues were calculated on the basis of 32 leucine residues/mol of enzyme (Ogawa & Fujioka, 1978). Each number represents the average from two or more determinations. [b] The reaction with butanedione was terminated when the residual activity was <5%. [c] These values include both free and amidated residues.

Table II: Effect of Butanedione, *p*-(Chloromercuri)benzoate, and Dithiothreitol on Saccharopine Dehydrogenase Activity[a]

treatment	% of initial act.
none	104
butanedione	6
butanedione + dithiothreitol	8
p-(chloromercuri)benzoate	0
p-(chloromercuri)benzoate + dithiothreitol	90
butanedione + *p*-(chloromercuri)benzoate	0
butanedione + *p*-(chloromercuri)benzoate + dithiothreitol	12

[a] Saccharopine dehydrogenase (0.7 nmol) was incubated with either butanedione (1.1 μmol) or *p*-(chloromercuri)benzoate (1.5 nmol), or with both, in 0.1 mL of Hepes-KCl-borate buffer. After incubation for 150 min at 25 °C, aliquots from the incubation mixtures were assayed for activity before and after addition of 0.05 mM dithiothreitol. The initial activity represents the activity before incubation. The activity of the native enzyme was not affected by dithiothreitol.

covered by treatment with dithiothreitol. We took advantage of this finding to exclude further the possibility that the inactivation by butanedione is due to the modification of the cysteine residue (Table II). The enzyme was incubated with butanedione together with *p*-(chloromercuri)benzoate. When the activity of the enzyme incubated with butanedione alone fell to ~6% of the initial value, an aliquot was removed from the incubation mixture, reactivated with dithiothreitol, and assayed for enzyme activity. The activity found was only 12% of the initial value rather than the 90% recovery for the enzyme incubated with *p*-(chloromercuri)benzoate alone. Although not conclusive, these results support the conclusion that a cysteine residue is not the site of modification.

Table I shows that a total of about eight arginine residues are lost in an almost completely inactivated enzyme. To estimate the number of residues responsible for activity, we examined a correlation between the loss of activity and the number of residues modified (Figure 3). Extrapolation of the initial portion of the curve to 0 enzyme activity yielded a value of approximately one residue. Thus, one arginine residue highly reactive toward the reagent is involved in the catalytic activity of the enzyme.

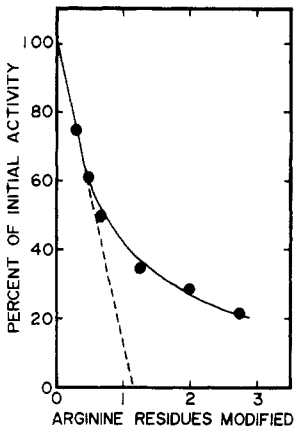

FIGURE 3: Correlation between residual enzyme activity and the number of arginine residues modified. Saccharopine dehydrogenase (1 mg/mL) was incubated with 11.2 mM butanedione. At various times, aliquots (0.1 mL) were removed from the reaction mixture, portions thereof were assayed for enzyme activity, and the remainders were added to 0.04 mL of 6 N HCl. Samples for amino acid analysis were prepared as described under Experimental Procedures.

Table III: Effect of Coenzyme and Substrates on Inactivation[a]

compd added (mM)	residual act. (%)
none	45
NAD^+ (1.0)	49
NADH (0.8)	46
α-Kg (10.0)	45
Lys (15.0)	45
Leu (20.0)	44
Sac (20.0)	42
NADH (0.8) + α-Kg (10.0)	47
NADH (0.8) + α-Kg (10.0) + Leu (0.5)	69
NADH (0.8) + α-Kg (10.0) + Leu (2.0)	80
NADH (0.8) + Sac (10.0)	84
α-Kg (10.0) + Leu (2.0)	49

[a] The enzyme (0.7 nmol) in 0.1 mL of Hepes-KCl-borate buffer was preincubated with the compounds at the concentrations indicated. 2,3-Butanedione (1.14 μmol, 5 μL) was then added, and the mixtures were incubated for 10 min at 25 °C. The residual activity represents the percentage of activity obtained by comparison with the uninhibited control. α-Kg, α-ketoglutarate; Lys, L-lysine; Leu, L-leucine; Sac, L-saccharopine.

Steady-State Kinetic Parameters of Modified Enzyme. The effect of modification on the steady-state kinetic parameters of the enzyme was examined with a 64% inactivated enzyme in both directions. The double-reciprocal plots of initial velocities against substrate concentrations were always linear with any of the reactants as the variable substrate, indicating that there were no enzyme species with different K_m values. The butanedione treatment altered only V_{max}, but not K_m, values (data not shown).

Effect of Coenzyme and Substrates on Inactivation. To test the effect of the coenzyme and substrates on inactivation, we incubated the enzyme with butanedione in their presence. Since NADH and NADPH are shown to be photooxidized by butanedione in borate or phosphate buffer (Homyk & Bragg, 1979), the incubation mixtures were protected from light as much as possible. As shown in Table III, neither the coenzyme nor substrates alone afforded protection even at saturating concentrations. L-Leucine, a competitive inhibitor of lysine (Fujioka & Nakatani, 1972), also exerted no protection. Whereas NADH and α-ketoglutarate in combination was without effect, a substantial protection was observed with leucine in their presence. Saccharopine dehydrogenase binds reactants in a compulsory order. The coenzymes (NAD^+ and NADH) are bound to the free enzyme, and in the direction

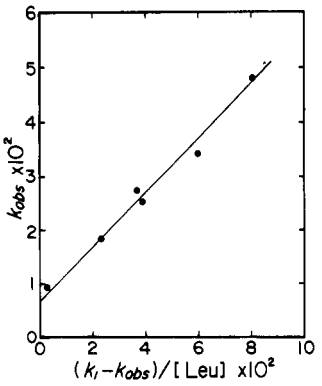

FIGURE 4: Protective effect of L-leucine on butanedione inactivation. The enzyme (0.7 nmol) was incubated with 11.4 mM butanedione in 0.1 mL of Hepes–KCl–borate buffer (pH 8.0) containing 0.8 mM NADH, 10.0 mM α-ketoglutarate, and various concentrations of L-leucine. The leucine concentrations were 0, 0.2, 0.5, 1.0, 2.0, and 20.0 mM. Values of k_i (pseudo-first-order rate constant in the absence of leucine) and k_{obsd} (those in the presence of leucine) were determined from the pseudo-first-order kinetic plots. See text for details.

of reductive condensation of lysine and α-ketoglutarate, α-ketoglutarate is the middle substrate that binds to the enzyme (Fujioka & Nakatani, 1970; Ogawa et al., 1979). Thus, the protection by leucine in the presence of NADH and α-ketoglutarate suggests that the modification by butanedione occurs on an arginine residue located at or near the binding site for lysine. That the protection by leucine is the result of a specific interaction at the active site is reinforced by the finding that saccharopine, a natural substrate, can give protection in the presence of NADH. A previous kinetic analysis has indicated that saccharopine forms an abortive complex with the enzyme·NADH complex (Sugimoto & Fujioka, 1978).

To ascertain whether leucine and saccharopine completely prevent the enzyme from inactivation, titration experiments were carried out with these compounds. (The titration mixtures with leucine and saccharopine contained saturating concentrations of NADH and α-ketoglutarate in the former and NADH in the latter). If it is assumed that the reaction of butanedione takes place also with the enzyme·NADH·α-ketoglutarate·leucine complex (or enzyme·NADH·saccharopine complex) and the modified enzyme is totally inactive, the apparent first-order rate constant for inactivation (k_{obsd}) in the presence of leucine (or saccharopine) may be given by

$$k_{obsd} = \frac{k_1 + k_2(L/K_D)}{1 + L/K_D} \quad (1)$$

which is rearranged to

$$k_{obsd} = (k_1 - k_{obsd})K_D/L + k_2 \quad (2)$$

where k_1 and k_2 are rate constants in the absence and presence of leucine (or saccharopine) (L), respectively, and K_D is the dissociation constant for the dissociation of leucine (or saccharopine) from the quaternary (or ternary) complex. Thus, a plot of k_{obsd} against $(k_1 - k_{obsd})/L$ should give a straight line with a definite vertical intercept (k_2). Figure 4 shows such a plot for leucine. A similar plot was obtained for saccharopine (not shown). As is evident from the figures, the protection was partial in either case. Values of the dissociation constant for leucine and saccharopine from the respective dead-end complexes (K_D) were determined from the slopes of these plots to be 0.51 mM and 0.54 mM, respectively. These values compare rather well with those obtained previously: K_D(leucine = 0.67 mM at pH 8.5 (Ogawa et al., 1979); K_i(saccharopine) = 0.2 mM at pH 7.25 (Sugimoto & Fujioka, 1978). The ratio k_1/k_2 was 9.9 for the leucine complex. The corresponding value for the saccharopine complex was 4.1.

Equilibrium Dialysis Study. Previous investigation has shown that the baker's yeast saccharopine dehydrogenase is a monomeric protein (Ogawa & Fujioka, 1978) and contains 1 binding site for reactants/mol of enzyme (Ogawa et al., 1979). As predicted by steady-state kinetic analyses (Fujioka & Nakatani, 1970; 1972) the binding study showed that α-ketoglutarate and leucine are bound only to the enzyme·NADH and enzyme·NADH·α-ketoglutarate complexes, respectively (Ogawa et al., 1979). Since the inactivation by butanedione is protected only by the amino acid substrate (or analogue), the binding capacity of the modified enzyme for the amino acid substrate was tested with [^{3}H]leucine as a radioactive ligand in the presence of saturating concentrations of NADH and α-ketoglutarate. The equilibrium dialysis study showed that the modified enzyme did bind [^{3}H]leucine with an apparent dissociation constant of 2.4 ± 0.5 mM. The corresponding constant for the native enzyme was 1.3 ± 0.3 mM. The latter value is ~2-fold greater than those found in the protection experiment (see above) and in the previous investigation (Ogawa et al., 1979) and appears to be less reliable because of a difficulty inherent to the method when the affinity of the ligand is low.

Discussion

In recent years, the monomeric 2,3-butanedione has been widely used in probing the functional role of arginine residues in catalytic mechanisms of many enzymes. The reagent has been shown to react in a highly selective manner with the guanido group of arginine in the model system and in proteins (Yankeelov, 1970; Riordan, 1973).

The present investigation shows that the baker's yeast saccharopine dehydrogenase is inactivated by butanedione. Although the abundance of arginine residues in the enzyme made it difficult to obtain definitive data, extrapolation of the initial portion of a curve relating the loss of activity and the number of arginine residues modified to 0 enzyme activity (Figure 3) indicates that one arginine residue is essential for activity. Previous studies have shown that the enzyme possesses an essential cysteine residue at the coenzyme-binding site (Ogawa et al., 1979) and essential histidine (Fujioka et al., 1980) and lysine (Ogawa & Fujioka, 1980) residues at the α-ketoglutarate-binding site, all of which are highly reactive toward chemical modification reagents. However, the finding that the contents of these functional residues are not seriously affected by butanedione treatment excludes the possibility that the inactivation is due to the modification of these residues. The experiment showing that the enzyme whose essential cysteine residue had been protected by *p*-(chloromercuri)benzoate could be inactivated irreversibly by butanedione (Table II) also supports the idea that the cysteine residue is not involved in inactivation. Thus, it is reasonable to conclude that the inactivation of saccharopine dehydrogenase by the diketone is the result of the reaction with an arginine residue.

The reaction of butanedione with arginine residues in proteins is usually stimulated by borate ion. Riordan (1973) has postulated that butanedione and an arginine residue reversibly form a dihydroxyimidazoline derivative and attributed the stimulatory effect of borate to the stabilization of the product through complex formation. With saccharopine dehydrogenase, however, borate did not stimulate the inactivation, nor was the activity recovered appreciably in the absence of borate. These phenomena are not without antecedent. Huang & Tang (1972), Pal & Colman (1976), and Ehrlich

& Colman (1977) reported irreversible inactivation resulting from the modification of arginine residues in the absence of borate. As pointed out by Riordan himself, secondary rearrangements of the proposed intermediate leading to irreversible loss of activity would be possible (Riordan, 1973).

The pH dependence of inactivation indicated the involvement of a group with a pK of 7.8. Since it is known that free arginine reacts readily with butanedione even at pH 7.5 (Riordan, 1973), this value does not appear to reflect the pK of an arginine residue. Rather the finding suggests the participation of another amino acid residue whose ionization makes the local environment more favorable for the reaction to take place or which assists the rearrangement of the cis diol adduct. Similar low pK values for inactivation are reported in carboxypeptidase (Riordan, 1973), alcohol dehydrogenase (Lange et al., 1974), and creatine kinase (Borders & Riordan, 1975).

Saccharopine dehydrogenase was largely protected from inactivation by leucine, a competitive analogue of lysine, in the presence of NADH and α-ketoglutarate, or by saccharopine in the presence of NADH. NAD^+, NADH, and α-ketoglutarate, either alone or in combination, were without effect (Table III). From the known order of addition of reactants to the enzyme, these results strongly suggest that the inactivation by butanedione is due to a specific modification of an arginine residue near the binding site for the amino acid substrate or, alternatively, that a subtle conformational change induced by the binding of the amino acid substrate renders the reaction of the reagent less favorable. Titration experiments showed that the protection by leucine and saccharopine is not complete and the reaction also takes place with the enzyme complexed with these ligands at a rate slower than with the free enzyme. Furthermore, the binding study revealed that the enzyme inactivated almost completely could still bind leucine in the presence of NADH and α-ketoglutarate, albeit less tightly than the native enzyme does. Thus, these results clearly indicate that the arginine residue does not participate directly in the binding of any of the reactants and suggest that the residue is involved in some way in the catalytic reaction that transforms substrates to products.

Although arginine residues have been shown to play essential roles in a number of pyridine nucleotide dependent dehydrogenases (Yang & Schwert, 1972; Adams et al., 1973; Foster & Harrison, 1974; Lange et al., 1974; Bleile et al., 1975; Blumenthal & Smith, 1975; Nagradova & Asryants, 1975; Austen & Smith, 1976; Levy et al., 1977; Pal & Colman, 1976; Ehrlich & Colman, 1977), there are considerable differences in their postulated roles. In dogfish M_4 lactate dehydrogenase (Adams et al., 1973) and alcohol dehydrogenase (Lange et al., 1974), it is proposed that the essential arginine residues are directly involved in the binding of the coenzyme. However, by binding studies, the coenzyme was shown to bind to the arginine-modified bovine H_4 lactate dehydrogenase (Yang & Schwert, 1972), cytoplasmic pig liver malate dehydrogenase (Bleile et al., 1975), yeast glyceraldehyde-3-phosphate dehydrogenase (Nagradova & Asryants, 1975), and *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Levy et al., 1977), even though the essential arginine residues occur apparently at the nucleotide sites. In pig heart $NADP^+$-dependent isocitrate dehydrogenase, an essential arginine residue is shown to be located in the region of the isocitrate-binding site (Ehrlich & Colman, 1977).

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