The Role of Arginine in the Triphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase of Pig Heart†

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ABSTRACT: The TPN-dependent isocitrate dehydrogenase from pig heart is inactivated by incubation with 2,3-butanedione at pH 6.5 and 25 °C. The rate of inactivation is linearly dependent upon butanedione concentration with a second-order rate constant of 0.059 M^{-1} min^{-1}. In the presence of manganous–isocitrate or isocitrate alone the rate constant for inactivation is reduced fivefold. The variation of the rate constant with isocitrate concentration permits calculation of binding constants for isocitrate alone (41 μM) and isocitrate in the presence of 2 mM manganous sulfate (6.5 μM) which are in agreement with previous direct measurements of the binding constants for isocitrate. In contrast to the protective effect of isocitrate, TPNH increases by a factor of 4.5 the rate of inactivation caused by butanedione. Amino acid analysis of acid hydrolysates of butanedione-modified enzyme reveals that loss of activity in the absence of additions is linearly related to modification of 10 arginines of the 24 in native isocitrate dehydrogenase. TPNH enhances the reactivity of some of these groups, producing inactivation concomitant with modification of only 4 arginine residues. This result is indicative of a conformational change produced by TPNH binding. Isocitrate decreases the rate of reaction of certain arginine residues, with loss of activity becoming proportional to modification of 20 arginines. Comparison of the extent of inactivation with the number of residues modified at a given time in the presence of various ligands reveals that the differences in the loss of activity may be ascribed to modification of 1–2 groups. Previous studies have shown that below pH 6 isocitrate dehydrogenase is inactivated by reaction of iodoacetate with a single methionine residue and that isocitrate protects against the alkylation. Prior modification of isocitrate dehydrogenase with butanedione decreases the incorporation of [^{14}C]iodoacetate into the enzyme, suggesting that selective modification of methionine in the active-site region is directed by interaction of the negatively charged reagent with positively charged arginyl residue(s). The results here presented indicate that inactivation of isocitrate dehydrogenase by 2,3-butanedione results from modification of arginine(s) in the region of the isocitrate binding site.

Elucidation of the amino acid residues which participate in the function of pig heart TPN^+[1]-dependent isocitrate dehydrogenase (*threo*-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) has been a concern of this laboratory for several years. Previous studies have implicated the presence of glutamyl (Colman, 1973), methionyl (Colman, 1968), and cysteinyl residues (Colman, 1969a) in the region of the active site. Isocitrate dehydrogenase has been shown to catalyze the oxidative decarboxylation of the complex of the negatively charged tribasic isocitrate with divalent metals (Colman, 1972a) and to bind isocitrate both in the absence and presence of the metal (Colman, 1969b). In the absence of metal the binding of tribasic isocitrate is independent of pH in the range from 5 to 8 (Ehrlich and Colman, 1976), indicating that the residues participating in the binding do not change their state of ionization in this pH region. Positively charged arginyl residues have been shown to be part of the binding site for several enzymes which have negatively charged substrates (Huang and Tang, 1972; Yang and Schwert, 1972; Riordan, 1973; Daemon and Riordan, 1974; Lobb et al., 1975; Riordan and Scandurra, 1975; Gilbert and O'Leary, 1975; Pal and Colman, 1976). Furthermore, in many dehydrogenases, an arginine residue has been located within the pyridine nucleotide coenzyme binding site (Lange et al., 1975; Foster and Harrison, 1974; Nagradova and Asryants, 1975). Since isocitrate dehydrogenase catalyzes a reaction involving both a negatively charged substrate and a pyridine nucleotide coenzyme, it seemed reasonable to explore the role of arginine residues in this enzyme in order to further delineate the nature of the substrate and coenzyme binding sites.

Butanedione has been used extensively as a reagent for the modification of arginine residues (Yankeelov, 1970; Huang and Tang, 1972; Yang and Schwert, 1972; Riordan, 1973) and these studies have indicated that other types of amino acid residues are either unaffected or react slowly (Yankeelov, 1970) under mild conditions. The present studies indicate that a maximum of four arginine residues can be implicated in catalytic activity and comparison of the differing rates of inactivation and of arginine modification in the presence of various ligands suggests that reaction with approximately one residue in the region of the isocitrate binding site is responsible for loss of enzyme activity.

Experimental Procedure

Materials. Pig heart isocitrate dehydrogenase without serum albumin was supplied by special arrangement from Boehringer Mannheim Corp. The preparation was further purified as previously described (Ehrlich and Colman, 1976). Purified enzyme was stored at −85 °C in 0.1 M triethanolamine chloride buffer (pH 7.7) containing 10% glycerol and 0.3 M sodium sulfate (standard triethanolamine buffer). Protein concentration was determined from the absorbance at 280 nm using $E_{280}^{1\%}$ = 9.1 (Colman, 1968) and a molecular weight of 58 000 (Colman, 1972b).

2,3-Butanedione, 99%, was purchased from Aldrich Chemical Co. Similar results were obtained when butanedione was used without further purification or after redistillation.

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[1] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TPN^+ and TPNH, triphosphopyridine nucleotide, oxidized and reduced forms, respectively.

Coenzymes and DL-isocitrate were obtained from Sigma Chemical Company. New England Nuclear supplied 1-^{14}C-labeled iodoacetic acid. This was diluted with nonradioactive iodoacetic acid from Mann dissolved in H_2O (pH 5.8) and purified by extraction with carbon tetrachloride.

Reaction of 2,3-Butanedione with Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.05–0.3 mg/mL) was incubated with 2,3-butanedione at 25 °C in 0.06 M Mes buffer (pH 6.5) containing 0.2 mM EDTA. In the absence of EDTA the rate of inactivation was variable, but reproducible results were obtained with EDTA concentrations of 0.005–0.2 mM.[2] During the course of reaction aliquots were assayed for isocitrate dehydrogenase activity in 0.03 M triethanolamine buffer (pH 7.4) containing 0.1 mM TPN^+, 2 mM manganous sulfate, and 4 mM DL-isocitrate. The reduction of TPN^+ to TPNH was followed at 340 nm using a Gilford 240 spectrophotometer with expanded scale recorder (0.1 absorbance full scale).

Amino Acid Analysis. Aliquots of the reaction mixture were removed at various times and diluted by one-third with 6 N HCl to halt the reaction and prevent regeneration of free arginine (Riordan, 1973). The precipitated enzyme was dialyzed overnight against 1 N HCl. The sample was then dried under vacuum in the presence of NaOH. The sample was then hydrolyzed in 6 N HCl in a tube which was flushed with nitrogen and then evacuated. Hydrolysis was at 110 °C for 22 h. Amino acid analyses were conducted using the short column of a Beckman 120C amino analyzer. Two to three analyses of each sample were performed with the average variation among analyses being 3%.

[^{14}C]Iodoacetate Incorporation into Native and Butanedione-Modified Isocitrate Dehydrogenase. Native isocitrate dehydrogenase was incubated at 25 °C with 1-^{14}C-labeled iodoacetate in 0.14 M Mes buffer (pH 5.4) containing 0.075 mM EDTA. In order to minimize any further reaction with iodoacetate during the subsequent treatment of the enzyme, 1 mM manganous sulfate and 2 mM DL-isocitrate were added at the end of the incubation period (Colman, 1968). The sample (0.4–1.1 mL) was centrifuged at 4 °C and then subjected to diafiltration at 4 °C with 10 mL of 0.1 M Mes buffer (pH 6.5) containing 10% glycerol and 0.3 M sodium sulfate in an Amicon Model 8MC ultrafiltration cell. The sample was then dialyzed overnight against 500 mL of standard triethanolamine buffer. Protein concentration of the modified enzyme was measured from the optical density at 280 nm. Iodoacetate incorporation was determined using the original specific activity of the iodoacetate and the counts per minute measured in 10 mL of ACS mixture (Amersham/Searle Corp.) with a Packard 3300 liquid scintillation counter. Butanedione-modified enzyme was prepared by incubation of native enzyme with butanedione in 0.04 M Mes (pH 6.5) until the activity was approximately 25% of its initial value. The sample was then diluted with an equal volume of 0.24 M Mes (pH 5.4) containing the iodoacetate. This sample was then incubated for the identical length of time as was the native enzyme and the subsequent treatment was also identical.

Results

Inactivation of Pig Heart TPN^+-Dependent Isocitrate Dehydrogenase by 2,3-Butanedione. Pseudo-first-order kinetics is observed for inactivation of isocitrate dehydrogenase to less than 20% of the initial activity for reaction in the presence of TPNH and to less than 30% of the initial activity in the absence of added ligands (Figure 1). In the presence of isocitrate the initial rate of inactivation may be described in terms of a single pseudo-first-order rate constant, but for periods greater than 2 h there is a considerable decrease in the rate of loss of activity.

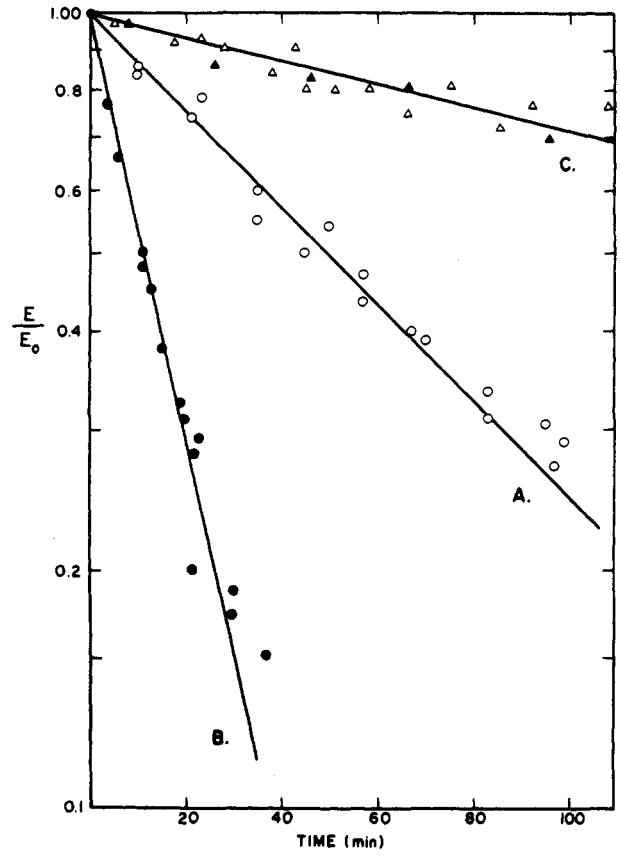

FIGURE 1: Inactivation of pig heart TPN^+-dependent isocitrate dehydrogenase (0.05 mg/mL) by 0.23 M 2,3-butanedione. Incubation was at 25 °C in 0.06 M Mes buffer (pH 6.5). The incubation mixtures include no additions (○), 100 μM TPNH (●), 20 mM DL-isocitrate (▲), or 2 mM manganous sulfate and 4 mM DL-isocitrate (△). Points from several experiments are included. The lines are used to calculate the pseudo-first-order rate constants.

The pseudo-first-order rate constant at 25 °C in the absence of added ligands was measured as a function of butanedione concentration. The variation of the observed rate constant with butanedione concentration is linear and the slope yields a second-order rate constant of 0.059 M^{-1} min^{-1}.

The effects of various ligands upon the rate of inactivation of isocitrate dehydrogenase by butanedione are illustrated by Figure 1 and the pseudo-first-order rate constants for 0.23 M butanedione are presented in Table I. Approximately a fivefold reduction in the rate of inactivation is obtained by incubation with isocitrate, either alone or in combination with manganous ion. Manganous ion, alone or with α-ketoglutarate, provides little protection against inactivation. A notable increase in the rate of inactivation occurs when TPN^+ or TPNH is included in the reaction mixture.

The rate of inactivation of isocitrate dehydrogenase by 2,3-butanedione in the presence of protecting ligands may be treated in terms of eq 1:

[2] It has been experimentally determined (R. S. Ehrlich and R. F. Colman, unpublished observations) that isocitrate dehydrogenase binds less than 0.05 mol of EDTA per mol of enzyme at 0.019 mM EDTA. Hence, it is likely that the low concentrations of EDTA act to chelate variable amounts of metal contaminants which may be present, thereby preventing their interaction with enzyme and improving the reproducibility of the results.

TABLE I: Rate of Inactivation of Isocitrate Dehydrogenase by 0.23 M 2,3-Butanedione in the Presence of Ligands.[a]

Additions to reaction mixture	k ($10^3 \times min^{-1}$)
None	13.6
2 mM Mn^{2+}	13.1
20 mM DL-isocitrate	2.6
2 mM Mn^{2+} and 20 mM DL-isocitrate	2.7
2 mM Mn^{2+} and 20 mM α-ketoglutarate	9.9
100 μM TPNH	61.0
100 μM TPN^+	21.0
200 μM TPN^+	33.2

[a] The pseudo-first-order rate constants were calculated from the slopes of graphs of log E/E_0 vs. time, as exemplified by Figure 1.

$$k_{app} - k_{min} = \frac{(k_0 - k_{min})}{\left(1 + \frac{[S]}{K_S}\right)} \quad (1)$$

where k_{app} is the observed pseudo-first-order rate constant; k_{min} is the rate constant at saturating levels of protecting ligand; k_0 is the rate constant in the absence of ligands; [S] is the concentration of protecting ligands; and K_S is the dissociation constant of the enzyme–ligand complex. Table II shows the decrease in the inactivation rate constant as the concentration of isocitrate is increased in the presence and absence of manganese. In the absence of manganese a value of 82 ± 18 μM DL-isocitrate is obtained for K_S. Expressed in terms of *threo*-Ds-isocitrate this value, 41 μM, is in reasonable agreement with the binding constant measured directly of 28 μM (Ehrlich and Colman, 1976). Applying eq 1 to the data on protection by isocitrate in the presence of 2 mM manganous sulfate a K_S of 13.1 ± 3.5 μM DL-isocitrate is calculated. This value, 6.5 μM if expressed in terms of *threo*-Ds-isocitrate, is somewhat higher than the comparable binding constant at pH 6.5 for *threo*-Ds-isocitrate in the presence of manganese (1.75 μM) measured by direct means (Ehrlich and Colman, 1975).

In most previous studies in which 2,3-butanedione was used as a reagent for the modification of arginine residues, borate was included in the reaction mixture to stabilize the product of reaction and prevent spontaneous restoration of activity upon removal of excess butanedione (Riordan, 1973). The addition of 0.11 M borate to the reaction mixture of butanedione and isocitrate dehydrogenase had no effect on the rate of reaction in the absence of ligands. The acceleration of the rate of inactivation in the presence of TPNH was not observed when borate was included. This result is presumably due to the formation of a complex between borate and the cis hydroxyl groups of the ribose adjacent to the nicotinamide ring of TPNH. Such a complex with DPN^+ and DPNH has been observed (Johnson and Smith, 1976; Smith and Johnson, 1976).

In order to assess whether the observed loss of activity upon treatment with 2,3-butanedione represented decreased affinity for substrate or coenzyme by an intrinsically active enzyme, isocitrate dehydrogenase inactivated in the absence of borate was diluted into standard triethanolamine buffer and was subjected to exhaustive dialysis against this buffer. No restoration of activity was observed at the end of this treatment. Enzyme diluted into standard triethanolamine buffer at 4 °C was therefore used to measure Michaelis constants for isocitrate and TPN^+. A Michaelis constant of 1.7 μM for isocitrate,

TABLE II: Rate of Inactivation of Isocitrate Dehydrogenase by 0.23 M 2,3-Butanedione in the Presence of Varying Isocitrate Concentrations.[a]

Concn (μM)	k ($10^3 \times min^{-1}$)	$K_{isocitrate}$ (μM)
DL-Isocitrate		
0	13.6	
37	10.7	103[b]
80	7.9	74[b]
160	5.9	69[b]
4 000	3.0	
20 000	2.6	Av 82 ± 18 μM
2 mM Mn^{2+} plus DL-isocitrate		
0	13.1	
9.3	9.6	18.3[c]
19	6.2	11.4[c]
37	5.0	10.6[c]
74	4.2	12.2[c]
4 000	2.9	
20 000	2.7	Av 13.1 ± 3.5 μM

[a] Pseudo-first-order rate constants have been corrected for loss of activity in controls which do not contain butanedione. [b] Calculated from eq 1 with $k_{min} = 2.6 \times 10^{-3}$ min^{-1} and $k_0 = 13.6 \times 10^{-3}$ min^{-1}. [c] Calculated from eq 1 with $k_{min} = 2.7 \times 10^{-3}$ min^{-1} and $k_0 = 13.1 \times 10^{-3}$ min^{-1}.

at pH 7.4, was found for enzyme with 24% of its initial activity, a value identical with that for a sample of native enzyme. The same partially active enzyme preparation exhibited a Michaelis constant of 10 μM for TPN^+ while the Michaelis constant for TPN^+ of native enzyme was 4 μM. From these results it is apparent that the inactivation cannot be ascribed to a large change in the Michaelis constants for isocitrate or TPN^+.

Modification of Arginyl Residues. The number of arginyl residues was measured by comparison with the measured quantity of histidyl and lysyl residues in the hydrolysates. The number of histidine residues was taken as 15 (Johanson and Colman, unpublished results) and 23.6 ± 0.4 arginines were found in native enzyme. The number of lysyl residues was measured as 46.4 ± 1.4 for native enzyme and 46.5 ± 1.3 for modified enzyme with 20–80% of the initial activity, indicating the absence of irreversible modification of lysyl residues. The ratio of histidyl to lysyl residues remained constant throughout the course of inactivation. The fluorescence spectra of native and butanedione-modified isocitrate dehydrogenase are identical, indicating that there is no appreciable reaction at tyrosine or tryptophan residues.

The progressive loss of arginine residues accompanying the time-dependent inactivation of isocitrate dehydrogenase by butanedione is shown in Figure 2. In the absence of additions, inactivation is proportional to the observed loss of arginyl residues (line A) with an extrapolated value of ten residues lost for complete inactivation. This result implies that all 24 arginines in isocitrate dehydrogenase are not identical in reactivity and some are more susceptible to attack by 2,3-butanedione than others. Addition of TPNH (line B) selectively increases the reactivity of certain residues and extrapolation shows that a modification of a maximum of four arginyl residues yields a total loss of enzymatic activity. In contrast, in the presence of manganous ion and isocitrate almost all arginines exhibit comparable reactivity; extrapolation of these data (line C) shows that 19.5 residues are modified out of a total of 24 at the

TABLE III: Number of Arginine Residues of Pig Heart TPN-Dependent Isocitrate Dehydrogenase Modified after Incubation with 2,3-Butanedione.[a]

	Addition to incubation mixture						
	(1) 100 μM TPNH		(2) 2 mM Mn^{2+} + 4 mM DL-isocitrate		Difference, (1) − (2)		No. of Arg modified for 100% inact.[b]
Time (min)	Inact. (%)	No. of Arg modified	Inact. (%)	No. of Arg modified	Inact. (%)	No. of Arg modified	
7	37	1.10	4	0.65	33	0.45	1.36
18	65	2.24	10	1.65	55	0.59	1.07
24	75	3.77	12	2.77	63	1.00	1.59
34	83	3.77	17	2.87	66	0.90	1.36
							Mean = 1.35 ± 0.21

[a] Isocitrate dehydrogenase (0.3 mg/mL) was incubated with 0.23 M 2,3-butanedione for the time periods indicated in the presence of various ligands. The incubation mixtures were quenched by addition of 6 N HCl and amino acid analyses were performed as described under Experimental Procedure. All results are averages from two or more analyses with the typical standard deviation being 0.3 residue. [b] Calculated from 100%/(difference in % inactivation) × difference in number of arginines modified.

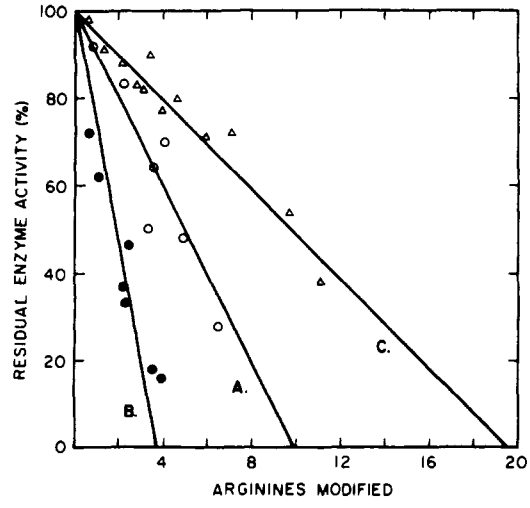

FIGURE 2: Residual enzyme activity as a function of the number of arginine residues modified. Reaction with butanedione was performed as in Figure 1 with no additions (○) (line A), 100 μM TPNH (●) (line B), or 2 mM manganous sulfate plus 4 mM DL-isocitrate (△) (line C) included in the incubation mixture. The lines showing the extrapolation of arginines modified to complete inactivation are linear least-squares fits to the data.

limit of zero enzyme activity (line C). The contrast suggests that the slow inactivation in the presence of manganous–isocitrate may be due to gross alteration of the enzyme structure, rather than to specific modification in the region of the active site.

It may be postulated that the observed number of arginines reacted at a given time consists of several residues whose reaction is unrelated to loss in activity or the presence of ligands, and a residue or residues that are required for activity and whose modification is affected by ligands. Additional insight into the number of arginine residues involved at the active site is thus obtained by comparing at the same incubation time the number of arginine residues modified and the percentage inactivation for samples incubated in the presence of different ligands (Table III). The comparisons are most readily made from the two Difference columns of Table III. For example, after 7 min, enzyme incubated with butanedione in the presence of manganous ion and isocitrate has lost 0.65 arginine residue, while enzyme incubated with TPNH has lost 1.10 arginine residues for a difference of 0.45 residue. The difference in percentage inactivation between these samples is 33%. If the difference in loss of arginine residues is linearly related to the difference in the percentage inactivation then complete loss of activity would require modification of 1.36 arginine residues based upon this particular set of data points. Examination of other incubation times indicates that 100% loss of enzyme activity can be related to modification of 1.35 ± 0.21 arginine groups. A similar comparison (at fixed incubation times) of data obtained in the absence of ligands with that in the presence of either TPNH or manganous ion and isocitrate gives 1.40 ± 0.50 arginine groups.

Further evidence for involvement of a small number of arginine residues was obtained from an experiment in which a sample was first incubated with butanedione in the presence of manganous–isocitrate for 120 min. This sample with 71% of the initial activity was split into two fractions. To one fraction a small volume of solution containing TPNH was added to yield a final TPNH concentration of 100 μM. An equal amount of buffer was added to the other fraction. The activity of the sample containing TPNH declined to 25% of the initial activity in 22 min while the other sample retained 62% of the activity of native enzyme; i.e., the two samples differed by 37% in the percentage inactivation. Amino acid analyses of the two samples failed to show a difference in arginine content of more than the experimental error of 0.4 residue. The rapid inactivation of the fraction containing TPNH suggests that an arginine whose modification leads to loss in activity is still susceptible to attack in the partially protected sample containing manganous–isocitrate.

Influence of Arginine Modification upon Incorporation of [1-^{14}C]Iodoacetate into Isocitrate Dehydrogenase. Isocitrate dehydrogenase is inactivated by alkylation of an average of one methionyl residue by iodoacetate (Colman, 1968). The rate of inactivation by iodoacetate has been found to be more rapid than the rate of inactivation by iodoacetamide (Colman, 1968). A possible explanation of the difference in the rate of alkylation is the presence of a positively charged site which initially binds iodoacetate. Since arginine may be involved in such a binding site the effect of modification of arginine residues upon the ability of isocitrate dehydrogenase to react with iodoacetate was examined. The results of iodoacetate incorporation studies are shown in Table IV. Inactivation of native isocitrate dehydrogenase by iodoacetate is shown by these results, as well as

TABLE IV: Incorporation of [1-^{14}C]Iodoacetate into Native and Butanedione-Modified Isocitrate Dehydrogenase.[a]

Enzyme preparation	Incubation time (min)	Inact. by iodoacetate (%)	Iodoacetate incorpd (mol/mol of enzyme)
Native	21	58	0.50
Butanedione-treated (25% active)	21	78	0.26
Native	50	81	0.73
Butanedione-treated (25% active)	50	90	0.36

[a] Native isocitrate dehydrogenase (1.3–1.4 mg/mL) was incubated with 63 mM [1-^{14}C]iodoacetate in 0.14 M Mes (pH 5.4) for the times indicated. Reaction was quenched by addition of 1 mM manganous sulfate and 2 mM DL-isocitrate. The sample was centrifuged and free iodoacetate removed as described under Experimental Procedure. Butanedione-modified enzyme was produced by incubation of native enzyme (2–2.5 mg/mL) with 0.2 M butanedione in 0.04 M Mes (pH 6.5) for 120 to 135 min to produce modified enzyme having approximately 25% of the initial activity. The modified enzyme was diluted 1:1 into 0.24 M Mes (pH 5.4) containing [1-^{14}C]iodoacetate at final concentrations of 63 mM iodoacetate and 0.14 M Mes. Subsequent sample treatment was the same as for native enzyme. The inactivation by iodoacetate is given as 100% minus the percent enzyme activity remaining after iodoacetate treatment as compared to enzyme activity prior to incubation with iodoacetate.

those of experiments using different iodoacetate concentrations, to be accompanied by the incorporation of 0.9 mol of [1-^{14}C]iodoacetate per mol of enzyme. These results are in agreement with previous studies (Colman, 1968). When iodoacetate was added to samples which had been partially inactivated (25% residual activity) by incubation with butanedione, the activity of aliquots from the mixture dropped rapidly by 30% within the first 5 min and then continued to lose activity at a higher rate than could be predicted from the rate of loss of activity of native enzyme. As can be seen from Table IV the incorporation of [1-^{14}C]iodoacetate into butanedione-treated isocitrate dehydrogenase is less than for native enzyme when both are incubated with iodoacetate for the same length of time. The small amount of incorporation of iodoacetate into butanedione-modified enzyme coupled with the rapid loss of activity suggests that most of the iodoacetate incorporated is bound to enzyme which does not have the arginine residue (or residues) required for activity modified by butanedione. Modification of arginine residues not directly responsible for activity must change the enzyme in such a way as to make the critical methionine more susceptible to attack by iodoacetate than in the native enzyme. The small total incorporation into butanedione-treated enzyme indicates that the rate of incorporation into butanedione-inactivated enzyme must be small. These results indicate that in the native enzyme unmodified arginine residues facilitate reaction of iodoacetate with the essential methionyl residue.

Discussion

The present work demonstrates that loss of arginine residues following incubation with 2,3-butanedione is related to loss of isocitrate dehydrogenase activity. Previous studies have indicated that 2,3-butanedione reacts in a highly specific manner with arginine residues (Riordan, 1973; Yankeelov, 1970). A 100-fold slower rate of reaction with lysine and an even slower rate of reaction with histidine residues has been observed (Yankeelov, 1970). In the current experiments it is unlikely that inactivation of isocitrate dehydrogenase is due to reaction with a lysine or histidine since the ratio of lysine to histidine residues remains constant as the enzyme is inactivated, while the ratio of arginine to histidine residues measured declines. Riordan has found that the rate of reaction of carboxypeptidase with butanedione is dependent upon the presence of borate and reversal of the reaction occurs upon removal of borate (Riordan, 1973). This phenomenon has been observed by other authors (Foster and Harrison, 1974) and has been taken as evidence for the involvement of arginine residues. The present studies have yielded no evidence for recovery of activity even in the absence of borate. Irreversible inactivation resulting from reaction of arginine residues in the absence of borate has been reported previously (Huang and Tang, 1972; Pal and Colman, 1976). Inclusion of borate in the incubation mixture is not desirable when enzymes using pyridine nucleotides are studied because of the formation of complexes between borate and the ribose moiety of the pyridine nucleotide (Johnson and Smith, 1976). Our results indicate that modification of arginine residues need not be correlated with an effect of borate on the rate of inactivation by butanedione or upon recovery of activity in the absence of borate. It is possible that internal secondary reactions could lead to formation of irreversibly modified arginine residues. Riordan (1973) has proposed a possible structure for the arginine–butanedione complex and its hydrolysis products but these have not been positively identified.

Direct measurements of the loss of arginine residues in acid hydrolysates of butanedione-treated enzyme indicate that the average number of residues affected depends upon the components of the incubation mixture. When no additions are made the number of residues modified upon extrapolation to complete loss of isocitrate dehydrogenase activity is ten. Inactivation is more rapid in the presence of TPNH but the number of residues lost when the enzyme becomes completely nonfunctional is four or less. Manganous–isocitrate substantially decreases the rate of inactivation by butanedione although its does not eliminate the inactivation. In the presence of manganous–isocitrate, complete inactivation corresponds to modification of at least 80% of the arginine residues in the enzyme. It is unlikely that such extensive alteration of amino acid residues will occur without gross changes in enzyme conformation; hence, inactivation in that case may be a result of these generalized changes rather than of specific site modification. Comparison of the number of arginine residues modified at given times in the presence and absence of effectors (Table III) indicates that activity may require the intact state of a single arginine residue. However, the possibility that these measurements involve merely the average of reaction at several sites, modification of any one of which results in loss of activity, cannot be excluded from the existent data.

The locus of the specific reaction producing loss of activity is probably within the isocitrate binding site since substantial protection is obtained in the presence of isocitrate. (However, the possibility remains that the protection against butanedione inactivation results indirectly from a conformational change produced by binding of isocitrate.) The reduction in the rate of loss of activity is the same with either isocitrate alone or manganous ion plus isocitrate but the concentration of isocitrate needed to achieve this protection decreases in the presence of manganous ion. This result is in agreement with direct binding measurements which show that the binding of isocitrate is strengthened by addition of manganous ion (Colman, 1969b). The isocitrate and manganous–isocitrate binding sites

are not identical since isocitrate dehydrogenase modified at cysteinyl or glutamyl residues binds isocitrate (and manganous ion) alone but fails to show the tightened binding of the manganous–isocitrate complex (Ehrlich and Colman, 1975). The present observation that protection may be achieved with either isocitrate or manganous–isocitrate provides evidence for overlap of the two sites. Inhibition by excess free isocitrate has been noted (Colman, 1972a). The role of arginine in the isocitrate binding site probably involves electrostatic interaction with one of the carboxyl groups of the substrate. Arginine has been implicated in the substrate binding site for negatively charged substrates in several enzymes (Riordan, 1973; Riordan and Scandurra, 1975; Gilbert and O'Leary, 1975; Pal and Colman, 1976).

One role for arginine in a number of enzymes has been shown to be participation in the binding site of pyridine nucleotides or pyridine nucleotide coenzymes (Lange et al., 1975; Foster and Harrison, 1974; Borders and Riordan, 1975). In the case of isocitrate dehydrogenase, the coenzymes, TPNH or TPN^+, fail to provide protection against enzyme inactivation by butanedione. On the contrary, the rate of inactivation is accelerated by inclusion of the coenzymes in the reaction mixture. It may be concluded that for isocitrate dehydrogenase arginine residues accessible to reaction with butanedione are not located within the nucleotide binding sites. Direct binding experiments have provided evidence that TPNH and manganous–isocitrate bind in a mutually exclusive fashion to isocitrate dehydrogenase (Ehrlich and Colman, 1975). The competition between TPNH and manganous–isocitrate might arise from binding to mutually exclusive but distinct sites or, alternatively, could arise from overlap of sites. The possible overlap of sites cannot be complete since the current experiments indicate that the critical arginine(s) susceptible to butanedione lies within the isocitrate site, but not the TPNH site. The enhanced reactivity of this arginine when TPNH is bound provides support for an interaction between the coenzyme binding site and the isocitrate site. Since TPNH actually enhances the reactivity of several arginine residues, the interaction observed could arise from changes in enzyme conformation. Such a change in conformation has been previously proposed (Rose, 1960).

Butanedione modification of arginine residues in isocitrate dehydrogenase has been shown to reduce the rate of incorporation of ^{14}C-labeled iodoacetate into the enzyme. Previous studies (Colman, 1968) have shown that iodoacetate at pH 5.6 reacts with a methionine residue with consequent loss of enzyme activity. A tenfold reduction in the rate of enzyme inactivation by iodoacetate is obtained when saturating concentrations of isocitrate or manganous–isocitrate are included in the reaction mixture. From a study of the variation of reaction rate with iodoacetate concentration it was previously concluded that iodoacetate binds weakly to the substrate binding site before reacting with the methionyl residue and inactivating the enzyme (Colman, 1968). The current results imply that intact arginine residue(s) may be part of the binding site for iodoacetate. Lange et al. (1975) have found that incorporation of iodoacetate into horse liver alcohol dehydrogenase is dependent upon the existence of an intact arginine residue. Iodoacetate reacts selectively with a single cysteine residue of alcohol dehydrogenase and reaction is more rapid than with iodoacetamide. In that case the arginyl residue is in the coenzyme binding site while the cysteinyl residue attacked by iodoacetate is liganded to the active-site zinc. The selective reaction of iodoacetate with methionine in isocitrate dehydrogenase is analogous. The arginyl residue in isocitrate dehydrogenase appears to be within the substrate binding site. While the role of the methionyl residue attacked by iodoacetate has not been completely ascertained, binding studies have shown that isocitrate (Colman, 1969b) and manganese binding (Villafranca and Colman, 1972) are not greatly affected by iodoacetate incorporation while TPNH fails to bind to the modified enzyme (Ehrlich and Colman, 1975). These results imply close spatial relationship between the coenzyme and substrate sites. The effect of butanedione in decreasing the reaction of isocitrate dehydrogenase with iodoacetate supports the hypothesis of Vallee and Riordan (1969) that the enhanced reactivity of some active-site residues with general alkylating reagents arises from the unique juxtaposition of other residues in the active site and that this spatial arrangement of residues is crucial to the catalytic role of the protein.

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