Effect of Arginine Modification on the Catalytic Activity and Allosteric Activation by Adenosine Diphosphate of the Diphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase of Pig Heart†

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ABSTRACT: DPN-specific isocitrate dehydrogenase from pig heart is allosterically activated by ADP which reduces the K_m for isocitrate. Treatment of the enzyme with 2,3-butanedione causes inactivation and, more rapidly, a disappearance of the ability of ADP to increase the initial velocity of assays conducted at low isocitrate concentrations. At pH 6.2 and 30 °C in the absence of manganous ion the second-order rate constants for inactivation (k_i) and loss of ADP activation (k_{ADP}) are 0.24 and 1.8 M^{-1} min^{-1}, respectively. The two rates are decreased no more than two- to threefold by DPN, by the nonactivator GDP, or by either isocitrate or ADP in the absence of manganous ion. In contrast, both rates are reduced 10–50 times in solutions containing manganous ion and either isocitrate or ADP. Total protection is observed when isocitrate is present together with ADP and Mn^{2+}. From the dependence of the decrease in k_i on the concentration of isocitrate in the presence of 2 mM Mn^{2+}, a dissociation constant of 300 μM for total isocitrate was determined. This value, when expressed as free dibasic isocitrate, the actual substrate, is 15 times as great as the K_m for free dibasic isocitrate measured under similar conditions. The difference may reflect a requirement for coenzyme to promote efficient binding of isocitrate at pH 6. A study of the dependence of the decrease in k_i and k_{ADP} on the concentrations of ADP and Mn^{2+} suggests that free forms of both ADP and Mn^{2+} are required for maximum protection. Although both k_i and k_{ADP} are reduced by Mn^{2+} plus either isocitrate or ADP, the two ligands cannot be occupying a single site because the combination of both is far more effective than either alone. The reciprocal influence of each of these ligands on the binding site for the other is proposed to be responsible for the protection provided by either isocitrate or ADP against inactivation and loss of activatability by ADP. Arginine residues are lost as a function of time of incubation with butanedione, and approximately two arginines are modified per average subunit of 40 000 when ADP activation and enzyme activity are totally eliminated. In comparison to 80% inactivated enzyme which had lost 1.6 arginines, enzyme incubated for the same time with Mn^{2+} + isocitrate (17% inactivated) or Mn^{2+} + ADP (32% inactivated) lost only 1.0 and 0.6 arginine, respectively. The specificity of protection by Mn^{2+}, ADP, and isocitrate against changes in the kinetic properties of the enzyme and against loss of arginine indicates the importance of arginine residues for both the catalytic function and allosteric activation of DPN-dependent isocitrate dehydrogenase.

The DPN-dependent isocitrate dehydrogenase from pig heart [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] is an allosteric enzyme which is positively regulated by ADP (Cohen and Colman, 1972). The residues involved in the activity of this enzyme have been under investigation in this laboratory for several years. On the basis of chemical-modification studies, lysyl (Shen and Colman, 1975; Hayman and Colman, 1977), cysteinyl (Mauck and Colman, 1976), and glutamyl or aspartyl residues (Ramachandran and Colman, 1977) have been determined to be critical to the function of isocitrate dehydrogenase. The positively charged arginine residue has been implicated in the binding of a variety of negatively charged compounds (Riordan, 1973; Riordan et al., 1977), and a number of investigators have established that arginine is involved in the substrate or pyridine nucleotide binding sites of dehydrogenases (Yang and Schwert, 1972; Riordan et al., 1977; Patthy and Smith, 1975; Bleile et al., 1975; Levy et al., 1977). Furthermore, there have been a few reports (Kantrowitz and Lipscomb, 1976; Riordan et al., 1977; Pal and Colman, 1976) indicating a role for arginine residues in the binding of nucleotides at regulatory sites. Since isocitrate dehydrogenase has a negatively charged substrate, coenzyme, and allosteric activator, it was considered likely that arginine residues would participate in the binding of at least some of these ligands.

2,3-Butanedione has been used as a specific reagent for arginine residues (Yankeelov, 1970; Riordan, 1973); under the usual conditions, reaction with other residues is either not detected or is slow relative to the rate with arginine (Yankeelov, 1970). The present study demonstrates that incubation of the DPN-dependent isocitrate dehydrogenase with 2,3-butanedione results in the modification of less than two arginines per average subunit and the loss at markedly different rates of both susceptibility to ADP modulation and of enzymatic activity. The interrelationship of the two types of sites can be deduced from the protection against loss of both ADP sensitivity and of enzymatic activity provided in the presence of manganous ion by either isocitrate or ADP.

Materials and Methods

Materials. The reagent, 2,3-butanedione, was Aldrich Red Label, 99%. Mes,[1] cellulose phosphate, ADP, DPN, DPNH, and sodium DL-isocitrate were purchased from Sigma

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN.

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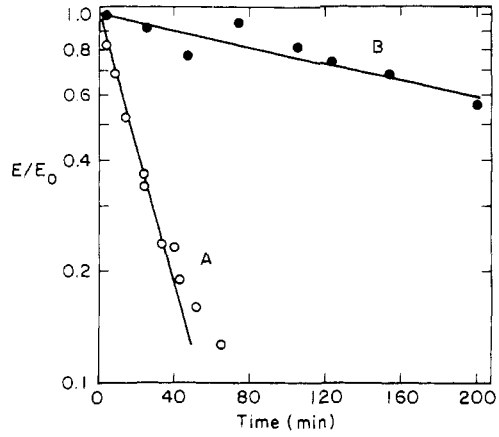

FIGURE 1: Inactivation of DPN-dependent isocitrate dehydrogenase by 0.2 M 2,3-butanedione at 30 °C in Mes buffer (pH 6.2). The detailed conditions of the incubation are described under Materials and Methods. At the indicated times, aliquots are withdrawn from the incubation mixture and assayed for isocitrate dehydrogenase activity in the presence of 20 mM isocitrate as described under Materials and Methods. The value E/E_0 is the ratio of the activity of the experimental enzyme sample to that of a control sample, which was identical except for the absence of 2,3-butanedione: (A) (○) 2.1 mM $MnSO_4$ was present in the incubation mixture: (B) (●) 2.1 mM $MnSO_4$ and 10 mM DL-isocitrate were present in the incubation mixture. The pseudo-first-order rate constants are 0.042 and 0.0026 min^{-1} for lines A and B, respectively.

Chemical Co.; DEAE-cellulose (DE-52) was from Reeves-Angel and Sephadex G-25 from Pharmacia. All other chemicals were reagent grade.

The DPN-dependent isocitrate dehydrogenase was purified from pig hearts by chromatography on DEAE-cellulose and cellulose phosphate, as described by Hayman and Colman (1977). Enzyme used for determination of the extent of arginine modification by amino acid analysis had a specific activity of 25 units/mg of protein. The DPN-dependent isocitrate dehydrogenase has a molecular weight of approximately 340 000 (Cohen and Colman, 1971) and appears to be composed of equal amounts of two distinguishable types of polypeptide chains (Ramachandran and Colman, 1978). However, the two subunit types have quite similar molecular weights, 39 000 and 41 000, and no information is as yet available about the possible functional significance of their diversity. Therefore, for the purposes of this paper, an average subunit molecular weight of 40 000 is used. Upon electrophoresis on polyacrylamide gels containing 2% sodium dodecyl sulfate, this preparation exhibited, in addition to less than 5% of slow-moving components, the two closely spaced bands that are characteristic of isocitrate dehydrogenase (Ramachandran and Colman, 1978).

Enzyme Activity and Protein Determinations. Enzyme activity was determined spectrophotometrically from the increase in absorbance of DPNH at 340 nm in a Gilford spectrophotometer, using a recorder set at 0.1 absorbance full scale. A 5-μL aliquot of enzyme was added to 1.0 mL of an assay solution (Cohen and Colman, 1972) containing 1.0 mM DPN, 1.3 mM $MnSO_4$, 20 mM DL-isocitrate, and Tris–acetate buffer (33 mM in acetate, pH 7.2). Protein concentration was estimated by multiplying the absorbance at 280 nm in a 1-cm light-path cuvette by 1.55 mg/mL (Shen et al., 1974). An enzyme unit is defined as that amount of enzyme which catalyzes the reduction of 1.0 μmol of DPN/min at 25 °C, and specific activity is expressed in units/mg of protein.

Kinetic Studies. The incubation solutions for inactivation by 2,3-butanedione were 50 mM in Mes (pH 6.2) and 20% in glycerol and contained the indicated concentrations of other components. The enzyme solution, which contained the same buffer with the addition of 2 mM $MnSO_4$, comprised 20% of the total volume of the incubation solution and was added immediately prior to the freshly diluted aqueous solution of 2,3-butanedione. The final protein concentration was about 0.2 mg/mL. All incubations and assays were carried out at 30 °C. For inactivation experiments, 5-μL aliquots of the incubation solution were removed at the indicated times for activity determinations as described above. When loss of ADP activation was followed, three assay solutions, all of which contained triethanolamine chloride buffer (36 mM in chloride) (pH 7.0), and 1 mM DPN, were used: (1) "standard assay", which was 20 mM in DL-isocitrate and 1.3 mM in $MnSO_4$; (2) "low isocitrate", which contained 0.5 mM DL-isocitrate and 1 mM $MnSO_4$; and (3) "ADP", which consisted of the low isocitrate solution plus 2 mM ADP. Assays of aliquots from a single incubation mixture were performed using the three solutions.

Michaelis constants for DL-isocitrate were determined in solutions containing 50 mM Mes (pH 6.2), 2 mM $MnSO_4$, 1.0 mM DPN, 0.2 M 2,3-butanedione, and 20% glycerol. The protein concentration was 0.7 μg/mL.

Measurement of Arginine Loss. Modification of arginine residues was followed by amino acid analysis of enzyme samples after reaction with 2,3-butanedione. Aliquots of reacted enzyme containing 0.2 mg of protein were diluted with an equal volume of 2 N HCl at 0 °C to stop the reaction and prevent regeneration of free arginine (Riordan, 1973). The modified enzyme was then dialyzed overnight at 4 °C against 1 N HCl. After the samples were dried in vacuo over solid NaOH, 1 mL of 6 N HCl was added and hydrolysis was performed as previously described (Shen et al., 1974). Measurements were made on the short column of a Beckman 120C amino acid analyzer using an expanded scale and normalizing arginine and lysine to the histidine composition of 10 mol per average polypeptide chain (Shen et al., 1974). Determinations were made in triplicate, and the average variation was ±4%.

Calculation of Concentrations of Free and Complexed ADP, Isocitrate, and Mn^{2+}. At pH 6.2, the total amounts of ADP and isocitrate present are distributed among the forms with three negative charges and with two negative charges as well as the corresponding metal chelates of these ionic species. The equation given in case I of Cohen and Colman (1972) (with, when appropriate, the substitution of ADP for isocitrate) was solved by means of a computer program for the various ionized species of free isocitrate, free ADP, free Mn^{2+}, and metal–ADP or metal–isocitrate complexes. The following constants were used for the calculations: the ionization constant for isocitrate, 1.79×10^{-6} M (Grzybowski et al., 1970); the association constant of Mn^{2+} and dibasic isocitrate, 57.5 M^{-1} (Grzybowski et al., 1970); the association constant of Mn^{2+} and tribasic isocitrate, 1150 M^{-1} (Grzybowski et al., 1970); the ionization constant for ADP, 3.98×10^{-7} M (Bock, 1960); the association constant of Mn^{2+} and ADP^{3-}, 23 400 M^{-1} (Colman, 1972); the association constant of Mn^{2+} and ADP^{2-}, 604 M^{-1} (Colman, 1972); the association constant of Mn^{2+} and SO_4^{2-}, 139 M^{-1} (Atkinson and Kor, 1965).

Results

Inactivation by 2,3-Butanedione. The DPN-dependent isocitrate dehydrogenase is inactivated by treatment with 2,3-butanedione, as exemplified by the pseudo-first-order plot shown in Figure 1, line A. The semilog plots are usually linear for at least two half-lives but then curve off. Ultimately, the enzyme activity is decreased to less than 1% of its initial value. Dramatic protection is provided by 10 mM isocitrate and 2

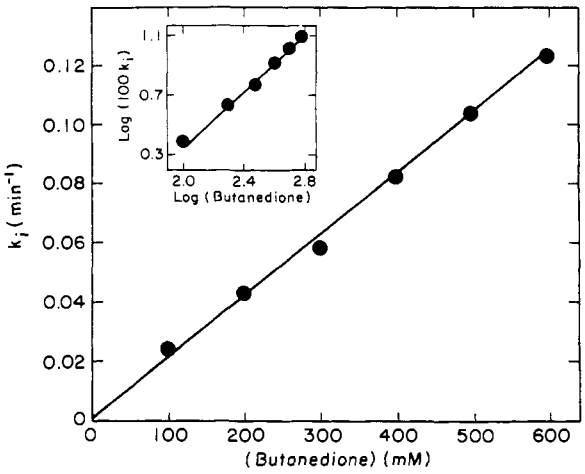

FIGURE 2: Dependence of k_i on reagent concentration. The incubation conditions are described under Materials and Methods. The incubation mixtures all contained 2.1 mM $MnSO_4$ and 2,3-butanedione as indicated. The rate constant for each reagent concentration was determined from a plot such as that shown in Figure 1, line A. The second-order rate constant is 0.21 M^{-1} min^{-1}. The inset shows a plot of log k_i vs. log [butanedione], with a slope of 0.95.

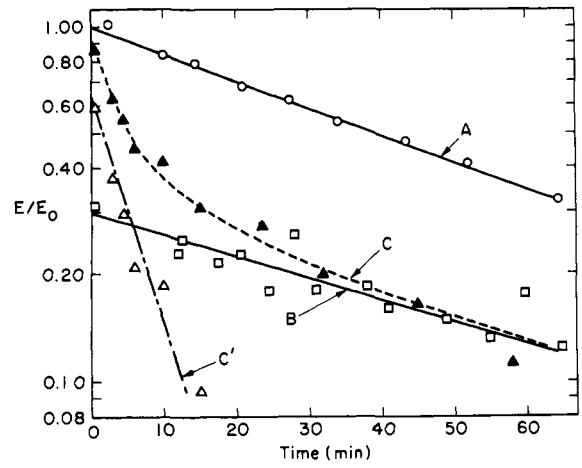

FIGURE 3: Loss of enzymatic activity and of ADP activation caused by treatment with 0.1 M 2,3-butanedione. The incubation solution contained 2.1 mM $MnSO_4$. At the indicated times, aliquots were withdrawn and assayed either at 20 mM DL-isocitrate (line A,○), at 0.5 mM DL-isocitrate (line B, □), or at 0.5 mM DL-isocitrate plus 2 mM ADP (line C, ▲). All rates are corrected for the loss of activity of suitable controls and are expressed as a fraction of the rate obtained at 20 mM isocitrate. Line C′ (△) was generated by subtracting E/E_0 for line B from the E/E_0 measured for line C at a given time. The pseudo-first-order rate constants are 0.017 min^{-1} for line A, 0.014 min^{-1} for line B, and 0.15 min^{-1} for line C′.

mM $MnSO_4$ (Figure 1, line B). The pseudo-first-order rate constant in the absence of ligands (k_i) is linearly dependent on reagent concentration (Figure 2), yielding a second-order rate constant of 0.21 min^{-1} M^{-1}. A plot of log k_i vs. log [butanedione] has a slope of 0.95 (Figure 2, inset).

Loss of Activation by ADP. The DPN-specific isocitrate dehydrogenase is allosterically activated by ADP which lowers the K_m for substrate but does not affect the maximum velocity (Cohen and Colman, 1972). The effect of ADP is conveniently observed as an increase in initial velocity caused when the nucleotide is added to solutions containing nonsaturating concentrations of isocitrate. In triethanolamine chloride buffer (pH 7.0), the addition of 2 mM ADP produces an increase of about threefold in the initial reaction velocity catalyzed by the native enzyme with 0.5 mM isocitrate, as represented by a comparison between the zero time points of Figure 3, lines B and C. Because reaction with 2,3-butanedione results in a loss of enzymatic activity, any effect of the reagent on regulation by ADP would be observable only under the condition that reduction in ADP activation occurs more rapidly than overall inactivation. Figure 3 shows the effect of 2,3-butanedione on the initial velocity measured at low isocitrate in the absence (line B) and presence (line C) of 2 mM ADP. A comparison between these lines indicates that the ability of ADP to lower the K_m for isocitrate is rapidly eliminated by treatment with 2,3-butanedione. Line A compares the decrease in maximum velocity of the same enzyme sample as measured at high isocitrate concentration. After 30 min, when the enzyme has lost about one-third of its initial activity, ADP has no effect on the initial rate of assays at a low level of isocitrate. At the beginning of the incubation period, the effect of the addition of ADP to 0.5 mM isocitrate assays is both to accelerate and to linearize the rate to one resembling the rate obtained at saturating levels of isocitrate. As inactivation by the reagent proceeds, the appearance of the "ADP" assay becomes similar to that with low isocitrate. This change is consistent with the abolition of the reduction of the isocitrate K_m by ADP. The "end point" of loss of ADP activation is an assay rate identical to the low isocitrate rate. Therefore, the ADP rate data are corrected by subtracting that fraction of the measured activity attributable to the unactivated reaction; that is, line B is subtracted from line C to generate C′, in Figure 3, which yields the corrected rate of loss of ADP activation. In most cases, the correction produces a plot that is pseudo-first-order for at least two half-lives, and the rate of loss of ADP activation (k_{ADP}) can be calculated from the line.

TABLE I: Effect of Ligands on the Rates of Enzyme Inactivation (k_i) and of Loss of Activation by ADP (k_{ADP}) Caused by 2,3-Butanedione.[a]

additions to reaction mixture[b]	k_i (min^{-1} M^{-1})	k_{ADP}[c] (min^{-1} M^{-1})
(1) none	0.24	1.8
(2) 0.4 mM $MnSO_4$	0.23	1.8
(3) 2.1 mM $MnSO_4$	0.21	1.3
(4) 50 mM sodium borate + 2.1 mM $MnSO_4$	0.20	2.1
(5) 10 mM DL-isocitrate	0.14	0.52
(6) 10 mM DL-isocitrate + 2.1 mM $MnSO_4$	0.015	0.030
(7) 5 mM ADP	0.10	0.65
(8) 5 mM ADP + 2.1 mM $MnSO_4$	0.030	0.065
(9) 5 mM GDP + 2.1 mM $MnSO_4$	0.12	0.78
(10) 1 mM DL-isocitrate + 5 mM ADP + 2.1 mM $MnSO_4$	0.006	0.008
(11) 3 mM DPNH + 2.1 mM $MnSO_4$	0.27	0.70[d]
(12) 2 mM DPN + 2.1 mM $MnSO_4$	0.10	0.73

[a] The inactivation procedure is described under Materials and Methods. The rate constants are obtained from data typified by Figure 1 and are expressed as second-order rate constants. [b] When $MnSO_4$ was omitted from the incubation solution, 1 mM EDTA was added to chelate the 0.4 mM $MnSO_4$ contributed by the enzyme solution. [c] The method of calculating k_{ADP} is explained in the legend to Figure 3 and exemplified by lines C and C′ of Figure 3. [d] This rate was not corrected for end point because in the absence of ADP DPNH so strongly inhibited the reaction at low isocitrate concentration that no rate could be measured.

Influence of Ligands on k_i and k_{ADP}. Table I lists a variety of compounds that affect the rates of enzyme inactivation and loss of activatability by ADP. $MnSO_4$ (Table I, lines 2 and 3)

TABLE II: Determination of the Dissociation Constant for Isocitrate (K_{IC}) from the Effect of Isocitrate on the Rate of Inactivation by 2,3-Butanedione.[a]

isocitrate (mM)	k_i (min^{-1} M^{-1})	K_{IC} (mM)
0	0.210	
0.15	0.128	0.28
0.25	0.140	0.45
0.30	0.105	0.34
0.50	0.076	0.22
0.75	0.058	0.21
1.0	0.055	0.34
5.0	0.020	
10.0	0.015	
		av 0.31

[a] All incubation solutions contained 2.1 mM $MnSO_4$. K_{IC} was calculated from eq 1, using $k_{min} = 0.015$ min^{-1} M^{-1}.

reduces k_{ADP} by only 30% while exerting very little effect on k_i. The acceleration by borate of enzyme inactivation by 2,3-butanedione is frequently considered diagnostic of a reaction with arginine residues (Riordan, 1973; Riordan et al., 1977). However, the inactivation of isocitrate dehydrogenase is unaffected by borate, whereas k_{ADP} is somewhat increased (line 4). The absence of a marked borate effect may be due to the fact that these studies were carried out at pH 6 where borate exists predominantly in its protonated form. Lange et al. (1974) postulated that only the borate anion can complex with the butanedione–arginine adduct.

When added with manganous ion, isocitrate (Table I, line 6) causes a striking decrease of both rates. However, isocitrate in the absence of $MnSO_4$ (Table I, line 5) decreases both rate constants by only two- to threefold. These results are consistent with the previous analysis of the kinetics of the enzyme (Cohen and Colman, 1974), which suggested that the enzyme–metal–isocitrate complex is formed either by reaction of the free enzyme with the preformed metal–isocitrate chelate or by reaction of the enzyme–metal complex with free isocitrate; no kinetic evidence was found for the formation of a specific enzyme–isocitrate complex.

The allosteric activator ADP when added together with metal ion also produces a marked decrease in both rate constants (Table I, line 8), although ADP alone causes only a two- to threefold lowering of the rate constants. The effect of ADP plus $MnSO_4$ appears to be specific, since the purine nucleotide GDP either with (Table I, line 9) or without $MnSO_4$ (data not shown) causes a decrease of only two- to threefold in both k_i and k_{ADP}. It has previously been shown that GDP does not allosterically activate the enzyme (Cohen and Colman, 1972). It appears that a variety of ligands can cause a relatively minor reduction in both rate constants as a result of weak and nonspecific binding which may produce small conformational changes in the protein. However, only isocitrate or ADP plus $MnSO_4$ effects decreases of 10- to 60-fold in the rate constants observed with 2,3-butanedione. It is notable that neither ligand causes total protection against inactivation. The combination of ADP and isocitrate together with manganous ion (Table I, line 10) produces a synergistic decrease in the rate constants and at higher concentrations prevents inactivation.

Although arginine has been found in the case of several enzymes (Lange et al., 1975; Foster and Harrison, 1974; Nagradova and Asryants, 1975; Blumenthal and Smith, 1975) to participate in the binding of pyridine nucleotide coenzymes, neither DPN nor DPNH (Table I, lines 11 and 12) provides marked protection against inactivation of isocitrate dehydrogenase by 2,3-butanedione, thus suggesting that the amino acid residues modified do not lie within the coenzyme sites. Indeed, DPNH actually causes a slight increase in k_i, while effecting the general two- to threefold decrease in k_{ADP}.

Reversibility of Inactivation by 2,3-Butanedione. It has been reported that the adduct between arginine and 2,3-butanedione is unstable in the absence of borate (Riordan, 1973). In order to ascertain whether isocitrate dehydrogenase could be reactivated after reaction with 2,3-butanedione, the following experiment was conducted. Enzyme was incubated with 0.1 M 2,3-butanedione in Mes buffer containing 2.1 mM $MnSO_4$ for 32 min at which time it had lost 60% of its original activity. This partially inactive enzyme was then subjected to gel filtration at 4 °C on a Sephadex G-25 column that had been equilibrated with 50 mM Mes (pH 6.2), containing 20% glycerol, 2.1 mM $MnSO_4$, and, for stabilization, the substrate 1 mM isocitrate. The specific activity of the enzyme decreased by two-thirds after the column. No recovery, but rather a gradual decline, of catalytic activity occurred during subsequent incubation for 2 h at 0 °C. Unless the dissociation of the reagent is obscured by the extreme lability of partially modified enzyme, the reaction of 2,3-butanedione with isocitrate dehydrogenase appears to be irreversible.

Determination of the Dissociation Constant for Isocitrate. The reduction of k_i caused by increasing concentrations of DL-isocitrate is shown in Table II. Even the highest isocitrate concentration, 10 mM, failed to prevent totally the inactivation of the enzyme. A dissociation constant (K_{IC}) was calculated for isocitrate by use of the following equation (Ehrlich and Colman, 1977):

$$(k_i - k_{min}) = \frac{(k_0 - k_{min})}{\left(1 + \frac{[IC]}{K_{IC}}\right)} \quad (1)$$

where k_i is the observed rate constant for inactivation of the enzyme in the presence of a given concentration of isocitrate, k_{min} is the minimal rate constant observed at high isocitrate, k_0 is the rate constant without isocitrate (but with 2.1 mM manganous ion), and [IC] is the isocitrate concentration. An average K_{IC} of 0.31 mM was obtained. In contrast, the K_m for isocitrate determined under similar conditions in Mes buffer (pH 6.2) was 0.014 mM. The corresponding values for dibasic isocitrate, the functional substrate (Cohen and Colman, 1972), are 35 and 1.4 μM for the K_{IC} and K_m, respectively.

Dependence of k_i and k_{ADP} on ADP and Manganous Ion. As was the case with isocitrate, total protection could not be achieved by increasing the ADP level, as shown in Table III. When $MnSO_4$ was held constant at 2.1 mM and the ADP concentration was gradually increased (Table III, lines 3–13) to 5 mM, both rates decreased; however, at ADP concentrations above 5 mM, the rates increased again. These results suggested that two different species of ligands were required for protection and that the minimum rate observed was attributable to an optimization of the concentrations of both species. In an attempt to clarify the mechanism of protection, the populations of ADP, Mn-ADP, and Mn^{2+} were calculated. It may be seen (Table III, lines 3–13) that, while free ADP increases continuously as the total ADP concentration is increased, the free manganous ion concentration decreases, particularly above 2 mM total ADP. These results are consistent with the requirement for both free ADP and free Mn^{2+} for maximum protection against inactivation and loss of ADP activatability. Alternatively, the total ADP concentration can

TABLE III: Dependence of k_i and k_{ADP} on the Concentrations of ADP and $MnSO_4$.

	total ligand concn		calcd concn of species of ADP and Mn^{2+}			rate constants	
line	$[ADP]_t$ (mM)	$[MnSO_4]_t$ (mM)	[Mn-ADP][a] (mM)	free [ADP] (mM)	free $[Mn^{2+}]$ (mM)	k_i (min^{-1} M^{-1})	k_{ADP} (min^{-1} M^{-1})
1	0	0	0	0	0	0.24	1.8
2	0	2.1	0	0	2.1	0.21	1.3
3	0.2	2.1	0.187	0.0132	1.53	0.15	1.4
4	0.5	2.1	0.462	0.0382	1.30	0.13	0.65
5	0.75	2.1	0.684	0.0659	1.12	0.15	0.55
6	1.0	2.1	0.898	0.102	0.942	0.14	0.44
7	1.5	2.1	1.28	0.218	0.631	0.080	0.16
8	2.0	2.1	1.57	0.426	0.397	0.055	0.13
9	3.0	2.1	1.86	1.14	0.174	0.045	0.085
10	4.0	2.1	1.95	2.05	0.102	0.041	0.075
11	5.0	2.1	1.99	3.01	0.0710	0.030	0.065
12	10.0	2.1	2.04	7.96	0.0276	0.055	0.095
13	20.0	2.1	2.06	17.9	0.0124	0.085	0.200
14	5.0	0.4	0.390	4.61	0.00910	0.050	0.32
15	5.0	2.1	1.99	3.01	0.0710	0.030	0.065
16	5.0	7.1	4.63	0.372	1.34	0.075	0.175
17	10.0	2.1	2.04	7.96	0.0276	0.055	0.095
18	10.0	7.0	6.59	3.41	0.208	0.048	0.17
19	10.0	12.1	9.17	0.828	1.19	0.040	0.11
20	20.0	2.1	2.06	17.9	0.0124	0.085	0.200
21	20.0	20.0	17.4	2.57	0.729	0.048	0.070

[a] The method of calculating the various species of Mn^{2+} and ADP is described under Materials and Methods. Free ADP is the total of ADP^{3-} and ADP^{2-}, while Mn-ADP is the total of the metal chelates of these two ionic species of ADP.

be maintained constant while the $MnSO_4$ concentration is increased. As shown in lines 14–16 of Table III, at a total ADP concentration of 5 mM, the rates decrease and then increase as the metal concentration is raised. The minimum rate constants are observed at a total Mn^{2+} concentration of 2 mM. At 0.4 mM $MnSO_4$ where free Mn^{2+} is only 0.0091 mM or at 17 mM $MnSO_4$ where the free ADP is only 0.372 mM, protection is diminished. It thus appears that significant concentrations of both free ADP and free Mn^{2+} must be present simultaneously for optimum protection. The data at 10 mM total ADP (Table III, lines 17–19) are less clear. However, the need for free Mn^{2+} is also supported by the data at 20 mM ADP (Table III, lines 20 and 21) where 20 mM $MnSO_4$ is distinctly more effective in lowering the rate constants than is 2 mM.

Modification of Arginine Residues. Modification of proteins by 2,3-butanedione has generally been ascribed to reaction with arginine residues, although this need not necessarily be the case. Determination of residual arginine groups after incubation with 2,3-butanedione was carried out in order to ascertain whether any loss of arginine had occurred and to quantify the number of arginines that had been modified. In no case was a loss of lysines observed. The relationship between inactivation and arginine loss is shown in Figure 4. The modification of arginine appears to be quite limited; the greatest loss that was measured under any condition was 1.6 residues out of a total of 13.4 per average subunit. Extrapolation of the line through the initial points to 0% residual activity yields a loss of 1.4 residues per average subunit. Modification of these residues must be responsible for loss of ADP activatability as well as for inactivation. After more than 60% of the initial activity is lost, there is an increasing loss of arginines, presumably as a result of nonspecific reactions.

Table IV shows the effect of ligands of the enzyme on both the modification of arginines and the inactivation of the enzyme caused by incubation with 2,3-butanedione for approximately 73 min. DPNH, which causes a slight increase in the

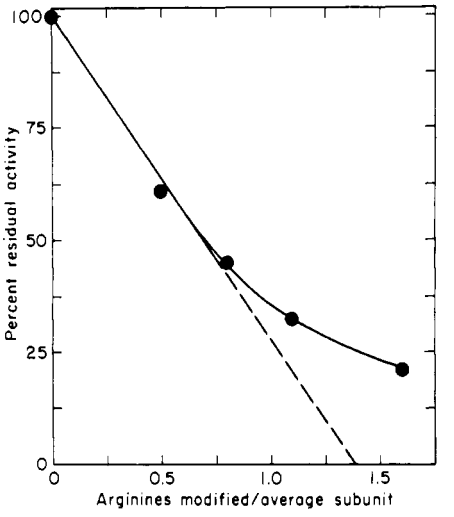

FIGURE 4: Relationship of arginine modification to loss of enzymatic activity. The enzyme was incubated for various times up to 73 min at 30 °C in 50 mM Mes (pH 6.2) containing 2.1 mM $MnSO_4$ and 0.1 M 2,3-butanedione. The details of the incubation, termination of reaction, and analysis of arginine modification are described under Materials and Methods. Unmodified enzyme had an arginine content of 13.4 residues per average subunit of 40 000 molecular weight.

rate of inactivation, does not significantly alter the extent of arginine modification. In contrast, when the enzyme reacts with 2,3-butanedione in the presence of manganous ion and either isocitrate or ADP (Table IV), the arginine loss is reduced by either 0.6 or 1.0 residue from that observed at the same incubation time. The results suggest that these ligands protect the arginine residues responsible for inactivation and loss of ADP activatability. In the case of ADP, the number of arginines modified corresponds reasonably well with the observed residual activity when compared with the curve generated in

TABLE IV: Change in Arginine Content in Enzyme Incubated with 2,3-Butanedione and Various Ligands.

addition to incubation solution	% residual act.	residual Arg/ av subunit	loss of Arg/ av subunit
none	100	13.4	
butanedione	21	11.8	1.6
butanedione + 3 mM DPNH	12	11.9	1.5
butanedione + 5 mM isocitrate	83	12.4	1.0
butanedione + 5 mM ADP	68	12.8	0.6
butanedione + 5 mM isocitrate + 5 mM ADP	100	11.9	1.5

[a] The enzyme samples were all incubated for approximately the same length of time (72.5–74.5 min) in a solution containing 50 mM Mes, pH 6.2, 20% glycerol, 2.1 mM $MnSO_4$, 0.1 M 2,3-butanedione and the ligands indicated. At the conclusion of the incubation period, the reactions were stopped by the addition of cold HCl as described in Methods. The percent residual activity was measured in comparison with the activity of control enzyme samples containing the same concentrations of the ligands, but lacking butanedione.

the absence of ligands (Figure 4); i.e., at 68% residual activity 0.4–0.5 arginine residue was modified in the absence of ligands as compared with 0.6 group in the presence of ADP. However, in the case of isocitrate, the number of arginines modified (1.0) is greater than the number reacted in the absence of ligands at the same residual activity (0.2 arginine residue at 83% residual activity). When isocitrate and ADP are added together, there is a greater decline in the number of arginine residues than when either is added separately. It has also been observed that very high isocitrate levels cause a greater arginine loss than occurs without any isocitrate.[2] It is possible that the enhanced binding of isocitrate caused by ADP may mimic the effect of a high isocitrate level on the enzyme conformation so as to render additional nonessential arginines reactive with 2,3-butanedione.

Discussion

The data presented in this paper support the conclusion that loss of both enzymatic activity and of the ability of ADP to activate the enzyme is occasioned by the reaction of 2,3-butanedione with a few arginine residues. Because these two processes occur at strikingly disparate rates, they must be reflective of independent reactions. The marked reduction in both rates that is provided by either isocitrate plus manganous ion or by ADP plus manganous ion suggests that the reaction is relatively limited and occurs at specific sites on the enzyme. It thus appears that discrete arginine residues are essential for catalytic function and for allosteric regulation of DPN-dependent isocitrate dehydrogenase.

The question then arises as to whether the sites attacked by 2,3-butanedione are actually the active site and the ADP-binding site. The involvement of a residue in an active site can be supported by the similarity of the dissociation constant of a ligand as determined from protection experiments, for example, K_{IC}, to the Michaelis constant of a substrate, with the caveat that the Michaelis constant may not be equivalent to the binding constant. In the present study, the K_{IC} when expressed in terms of the active substrate, free dibasic isocitrate, is 35 μM, a value 25 times as great as the K_m for free dibasic isocitrate of 1.4 μM measured under analogous conditions. In comparison, the ratio of K_{IC} to K_m for the inactivation of the enzyme by 2,4-pentanedione (Hayman and Colman, 1977) was 4, a value determined at the same pH but in Pipes buffer containing 0.44 M KCl. On the other hand, the ratio of K_{IC}/K_m of 25 determined in the present study is the same as that obtained by Mauck and Colman (1976) for the reaction of iodoacetate with DPN-isocitrate dehydrogenase at pH 6.0 in Pipes buffer containing 0.1 M KCl. These results might be interpreted to indicate that the residues attacked by iodoacetate, 2,3-butanedione, and 2,4-pentanedione lie within a second isocitrate binding site distinct from that involved in catalytic function. Indeed, Mauck and Colman (1976) postulated that such a distinct site had the function of maintaining the conformation of active enzyme. Alternatively, it is possible that isocitrate cannot bind efficiently at the active site in the absence of cofactor; i.e., ordered binding occurs. If this is the case, the dissociation constant measured for isocitrate (with manganous ion) in the absence of coenzyme, either directly or from protection against inactivation by a chemical reagent, would necessarily be higher than the K_m for isocitrate, which is determined in the presence of both manganous ion and coenzyme. Evidence that might be considered to contradict the postulate of ordered binding is the observation by Shen and Colman (1975) that isocitrate (with manganous ion) prevented inactivation of the enzyme at pH 7.4 by cyanate with a binding constant comparable to its K_m. However, this identity between the K_m and the K_d as measured by protection against inactivation has been observed only at pH 7.4. It may well be that the kinetic mechanism of the enzyme changes between pH 7 and 6, so that a random mechanism obtains at pH 7 but an ordered one exists at pH 6; if the mechanism is indeed ordered at pH 6, the K_{IC} may reflect binding in the active-site region of the enzyme.

A similar question may be raised with respect to the regulatory site; i.e., is the loss of allosteric sensitivity to ADP a result of reaction of an arginine in the region of the ADP binding site? Although extensive data are not available on the concentration dependence of activation by ADP, the concentrations of ADP affording effective protection against 2,3-butanedione (Table III) are similar in magnitude to those which lower the K_m for isocitrate (Cohen and Colman, 1972). In the present study, the data dealing with the dependence of the reduction of k_i and k_{ADP} on the concentrations of ADP and manganous ion may provide an insight into the question of which form of ADP, free or metal chelated, is the actual allosteric modifier. From these experiments, it can be concluded that free, rather than chelated, ADP binds and that a sufficient concentration of free Mn^{2+} is also required for optimal protection. The chelate seems to have no effect on the inactivation rates because (Table III, lines 9–13) when the chelate concentration is relatively constant the protection goes through a maximum and decreases.

With either Mn^{2+} and ADP or Mn^{2+} and isocitrate, protection against inactivation and loss of allosteric regulation are very marked (Table I, 7- to 15-fold for k_i; 30- to 60-fold for k_{ADP}); however, the rates of inactivation and loss of sensitivity

[2] Hayman, S., and Colman, R. F., unpublished observations.

to ADP activation are not decreased to zero. These results could be interpreted to suggest that these ligands are not binding directly at the sites attacked by butanedione but rather at adjacent sites. Thus, even if the ligand binding site is saturated, some reaction with 2,3-butanedione can still occur. In the case of ADP plus Mn^{2+}, the failure to obtain total protection of the enzyme may be due to the difficulty of providing the necessary simultaneous concentrations of both ligands. The failure to obtain total protection by isocitrate and Mn^{2+} may be related to the observation that isocitrate plus Mn^{2+} does not prevent arginine modification (Table IV), even when inactivation is almost totally prevented. Thus, it may be that 2,3-butanedione reacts with different arginines when isocitrate is present and that attack at these arginines causes the minor losses of activity and ADP sensitivity.

The relationship between the two types of sites attacked by 2,3-butanedione bears examination in view of the fact that both ADP and isocitrate cause the reduction of both k_i and k_{ADP}. These results may be interpreted as meaning that the allosteric and active sites are intimately interrelated. Fan et al. (1977) in a study of the reaction of histidine residues in bovine DPN-isocitrate dehydrogenase observed the loss of both enzymatic activity and ADP responsiveness. ADP without added manganous ion somewhat reduced the loss of regulation and activity, whereas manganous-isocitrate protected both activity and, to a lesser extent, ADP activation. These findings are consistent with the interactions observed with 2,3-butanedione inactivation. It is not unexpected that isocitrate and ADP would affect one another's binding sites. For ADP to cause a lowering in the Michaelis constant for isocitrate, it must cause an alteration in the conformation of the active site, and, undoubtedly, isocitrate also affects the binding of ADP. An alternative, but less likely, explanation is that neither ligand is binding directly to either the catalytic or allosteric site but rather to totally separate locations that control the conformations of the active and regulatory sites. That both ligands are not binding to a single site which is distinct from the active or allosteric site can be concluded from the total protection that is achieved only when both ADP and isocitrate are present with manganous ion. At present it is not possible to differentiate between the possibilities that by binding to its appropriate site either isocitrate or ADP can protect the other allosteric or active site or that either ligand is binding to a totally separate site and indirectly protecting both rates.

Other investigators have abolished allosteric effects by reaction with arginine residues. Kantrowitz and Lipscomb (1976) in a study of aspartate transcarbamylase observed that reaction with phenylglyoxal resulted in the loss of enzymatic activity, and that, when enzymatic activity was protected by a transition-state analogue, allosteric regulation was eliminated by treatment with phenylglyoxal. Riordan et al. (1977) reported that 2,3-butanedione modified fructose 1,6-diphosphatase, causing a rapid loss of inhibition by AMP and a slower loss of catalytic activity.

Arginine has been implicated in the coenzyme binding sites of several other dehydrogenases (Bleile et al., 1975; Foster and Harrison, 1974; Nagradova and Asryants, 1975; Austen and Smith, 1976), but the TPN-dependent isocitrate dehydrogenase from pig heart (Ehrlich and Colman, 1977) when reacted with 2,3-butanedione is modified in the substrate site, rather than the TPN site. For this enzyme, TPNH actually accelerates the rate of inactivation much more markedly than DPNH accelerates the rate of inactivation of the DPN enzyme. Because of the lack of specific protection by either DPN or DPNH in the present experiments, there does not appear to be any reaction of arginine residues in the region of the cofactor binding site of the DPN-isocitrate dehydrogenase.

Whenever enzyme-modification studies are carried out, it is important to ascertain both how many residues are reacted and how many reacted residues are essential to activity. A number of investigators (Marcus et al., 1976; Gawron and Jones, 1977) have used a plot of the log of $1/t_{1/2}$ vs. log [2,3-butanedione] as a method of estimating the order of the reaction with respect to reagent concentration; i.e., the number of arginines per active site modified to cause inactivation. Such a plot is illustrated in the inset to Figure 2. The linearity of the plot of k_i vs. reagent concentration makes it inevitable that the slope of the inset would be unity. The log-log plot appears to have been applied first to protein-modification studies by Levy et al. (1963) for the reaction of 2,4-dinitrophenol with myosin, and these authors cautioned that only a minimal order would be determined because some groups might be saturated by reagent over the range studied. Other conditions might be envisioned that would lead to an underestimate of the residues modified. For example, slow reaction of one residue might be succeeded by more rapid, kinetically unobservable, modification of an unknown number of residues which are essential to activity. Because of such uncertainties, a kinetic determination of stoichiometry is highly unreliable and is not an acceptable substitute for the chemical determination of the number of arginines modified.

In the present study, inactivation of the enzyme together with loss of susceptibility to ADP activation can be attributed to the reaction of approximately 1.4 arginines, about 10% of the number of arginine residues per average subunit. There are, of course, uncertainties in the determination of stoichiometry from the loss of a small number of residues such as is observed here. It is not known whether the measured loss is due to reaction at individual arginines rather than to the average of fractional reactions at many arginines, and the errors in the measurement of small differences are unavoidably large. In addition, the existence of two types of subunits and the absence of information about the numbers of active and regulatory sites in the oligimeric DPN-isocitrate dehydrogenase create an ambiguity about the relationship of the number of arginines lost to the active and allosteric sites. However, the correlation of the number of arginines lost in the presence of ADP to the loss of activity implies that, except when isocitrate is present, all the modification of arginines is specifically related either to activity or regulation. Isocitrate, in contrast, appears to facilitate the reaction of peripheral arginines with the reagent.

Notwithstanding the difficulties in interpretation that have been discussed, the simplest explanation of the findings of this study is that arginine residues are essential to the binding of both the substrate isocitrate and the allosteric regulator ADP, presumably in the active- and allosteric-site regions, respectively. The relatively small number of groups whose reaction results both in total inactivation and in loss of ADP sensitivity (1.4 per average subunit) makes it unlikely, although not impossible, that every subunit contains both an active site and a regulatory site, but this question must be further investigated by other techniques.

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