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Affinity Labeling of Nicotinamide Adenine Dinucleotide Dependent Isocitrate Dehydrogenase by the 2′,3′-Dialdehyde Derivative of Adenosine 5′-Diphosphate. Evidence for the Formation of an Unusual Reaction Product†

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ABSTRACT: Modification of the pig heart NAD-dependent isocitrate dehydrogenase by the 2′,3′-dialdehyde derivative of ADP (oADP) resulted in a time-dependent inactivation of the enzyme. Two kinetically distinct phases are observed for the loss in enzymatic activity with maximum rate constants of 0.38 and 0.023 min^{-1}, at saturating concentrations of oADP, at pH 7.0 and in the presence of 2.0 mM $MnSO_4$. The K_i values for both phases of the reaction are very similar; an average of 22.9 ± 4.6 μM for free oADP is obtained with constants determined in the presence of 0.2, 0.3, and 2.0 mM $MnSO_4$. At pH 7.0 and in the presence of Mn^{2+} ions, almost complete protection of isocitrate dehydrogenase from inactivation by oADP is provided by ADP and isocitrate, while only partial protection is afforded by NADH and ATP, and NAD is without effect. Only the protection by ADP is consistent with its directly determined binding constant which may indicate that isocitrate, NADH, and ATP exert allosteric effects on the inactivation by oADP, whereas ADP may compete with the analogue for the same nucleotide binding site. Affinity labeling of isocitrate dehydrogenase with [^{14}C]oADP results in radioactive labeling of the three distinct subunits. The incorporation of approximately 1 mol of [^{14}C]oADP/mol of average subunit corresponds to total inactivation of the enzyme. Inactivation of isocitrate dehydrogenase by oADP resulted in the formation of an enzyme–oADP product that was unaffected by subsequent reaction with sodium borohydride which suggests that the reaction product with this enzyme was not the generally expected Schiff base. Formation of the relatively stable product involved a loss of the pyrophosphoryl group of oADP as demonstrated by a comparison of the stoichiometry of the reaction determined with [^{14}C]oADP and [^{32}P]oADP. Further evidence obtained in this study is most consistent with the formation of a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative between oADP and the ε-amino group of lysine on isocitrate dehydrogenase. The results obtained through affinity labeling of NAD-dependent isocitrate dehydrogenase by oADP suggest that an allosteric site for ADP is present on each type of subunit and that the structurally distinct subunits of this enzyme may be functionally similar.

NAD-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating); EC 1.1.1.41] has an important role in the control of the mitochondrial oxidative decarboxylation of isocitrate to α-ketoglutarate (Plaut, 1970; Colman, 1975; Dalziel, 1980). The enzyme isolated from pig heart is composed of three distinct types of subunits in the approximate ratio 2α:1β:1γ (Ramachandran & Colman, 1980). Binding experiments indicated that the activators, Mn^{2+} and ADP, and the substrates, NAD and isocitrate, each have one binding site for every two subunits (Ehrlich & Colman, 1981). The stoichiometry of the ligand binding suggests either that isocitrate dehydrogenase has half the number of catalytic and ADP regulatory sites as it has subunits or that strong negative cooperativity exists in ligand binding. The latter assumption may be supported by the detection of enzymatically active α and β subunits by isoelectric focusing in the presence of 20% glycerol (Hayman & Colman, 1982) and by an initial report describing the isolation of catalytically active α and β subunits by chromatofocusing (Ehrlich & Colman, 1982a). Furthermore, modification of isocitrate dehydrogenase by 3-bromo-2-ketoglutarate, 3,4-didehydro-2-ketoglutarate, cyanate, and carbodiimide, which appear to label the substrate binding site, resulted in labeling of all types of subunits (Bednar & Colman, 1982). These results are consistent with the postulate that the structurally distinct subunits are functionally similar.

Isocitrate dehydrogenase is allosterically activated by ADP, which lowers the K_m for isocitrate (Cohen & Colman, 1972), as well as the K_m for Mn^{2+} (Cohen & Colman, 1974), but has no effect on the intrinsic maximum velocity. ADP appears

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to induce a metal-dependent aggregation of the NAD-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1981), in addition to causing activation. Binding studies have indicated the existence of a high-affinity binding site for free ADP on isocitrate dehydrogenase in the presence of Mn^{2+} ions (Ehrlich & Colman, 1981). For characterization of the allosteric site of ADP and for examination of its interaction with other sites of the enzyme, affinity labeling of the NAD-dependent isocitrate dehydrogenase was carried out in this study with the 2′,3′-dialdehyde derivative of ADP (oADP).[1] This structural analogue of ADP was also employed to further probe the function of the three types of subunits.

Experimental Procedures

Materials. The pig heart isocitrate dehydrogenase was prepared as previously described (Ramachandran & Colman, 1980; Ehrlich et al., 1981) except that dithiothreitol was omitted from the cellulose phosphate column buffer. The purified enzyme had a specific activity of 25–30 μmol of NADH formed min^{-1} (mg of enzyme)$^{-1}$.

NAD, NADH, ADP, ATP, and DL-isocitrate were obtained from Sigma Scientific Co. ^{32}P-Labeled inorganic phosphate, NaB^3H_4, [8-^{14}C]ADP, Econofluor, and Protosol were supplied by New England Nuclear Corp. ACS was obtained from Amersham, Pronase from Calbiochem, and carboxypeptidase A from Worthington Biochemical Corp. Aminopeptidase M and carboxypeptidase B were purchased from Pierce.

Enzyme Activity and Protein Determinations. Isocitrate dehydrogenase activity was measured spectrophotometrically at 340 nm in a Gilford spectrophotometer, using a recorder set at 0.1 absorbance full-scale. Assays were carried out at 25 °C in a standard assay mixture containing 1.0 mM NAD, 1.3 mM $MnSO_4$, 20 mM DL-isocitrate, and Tris–acetate buffer (33 mM in acetate, pH 7.2). The protein concentration of the native enzyme was measured spectrophotometrically at 280 nm by using a value of 6.40 for $E^{1\%}_{280}$ and a value of 40 000 for the average subunit molecular weight (Ramachandran & Colman, 1980). The protein concentration of modified isocitrate dehydrogenase was measured with the Bio-Rad protein assay which is based upon the method of Bradford (1976). Native isocitrate dehydrogenase was used as a standard.

Synthesis of oADP. Synthesis and purification of oADP were carried out as described by Easterbrook-Smith et al. (1976). The concentration of oADP was determined spectrophotometrically at 258 nm, using a value of 14 900 cm^{-1} M^{-1} for the extinction coefficient (Hansske et al., 1974). The purity of the analogue was confirmed by thin-layer chromatography on poly(ethylenimine) sheets, with 0.8 M NH_4HCO_3 as the developing solvent (Easterbrook-Smith et al., 1976), and by electrophoresis on cellulose plates, with pyridine/acetic acid/H_2O, pH 6.4 (25:1:250) as the buffer system. [8-^{14}C]-oADP was prepared from [8-^{14}C]ADP (1–3 Ci/mol) by the same procedure. [β-^{32}P]ADP (4.5 Ci/mol) was used as the starting material for the synthesis of [β-^{32}P]oADP. [β-^{32}P]-ADP was obtained from nonradioactive ADP by an exchange reaction with ^{32}P-labeled inorganic phosphate catalyzed by polynucleotide phosphorylase (Eliassson & Reichard, 1978).

Kinetics of Reaction of oADP with Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.25 mg/mL) was incubated at 25 °C in 50 mM Pipes buffer, pH 7.0, containing 20% glycerol and 2.0 mM $MnSO_4$. Under these conditions, a decrease in enzymatic activity of 10–15% is observed during the initial 30 min of incubation time. The activity then stabilizes and decreases by less than 10% during the following 9 h of incubation time. The enzyme was, therefore, incubated for 60 min prior to the addition of oADP. At the indicated times, aliquots were withdrawn and diluted 200-fold into the assay mixture described above. The enzymatic activity of the control incubation was taken as 100% residual activity (or E_0).

The above procedure describes the standard modification conditions; changes in either the concentration of $MnSO_4$ or the pH of the Pipes buffer to 6.1 are specifically noted in the text and legends.

Reduction by $NaBH_4$ at pH 7.0. Reduction of modified isocitrate dehydrogenase was carried out at 25 °C with a fresh solution of $NaBH_4$ in 10 mM NaOH. The reductant was added in three small aliquots (final concentration 30 mM, final pH 7.0) and at 5-min intervals to a modification reaction in Pipes buffer (50 mM, pH 7.0) containing the usual reaction components. So that the effectiveness of this procedure at pH 7.0 could be determined, a 0.2 mM solution of [^{14}C]oADP was reduced under identical conditions, followed by separation of the dialcohol derivative from [^{14}C]oADP by thin-layer chromatography on poly(ethylenimine) sheets (0.8 M NH_4HCO_3). The amount of each derivative was then determined by measuring the radioactivity associated with the two analogues. The results obtained showed complete reduction of oADP to the dialcohol under the conditions employed.

Incorporation of [^{14}C]oADP into Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.4 or 0.5 mg/mL) was inactivated by 0.2 mM [^{14}C]oADP (1–3 Ci/mol) as described above. At intervals, the enzyme was separated from excess reagent by filtration over two consecutive Sephadex G 50-80 columns (equilibrated with 50 mM Pipes, pH 7.0, containing 20% glycerol and 2.0 mM $MnSO_4$) by using a column centrifugation technique described by Penefsky (1979). The total separation time with this technique was under 7 min. Reduction of the modified mixture with $NaBH_4$ prior to the filtration step was without effect on the stoichiometry of the reaction and subunit labeling. The filtered protein was assayed immediately for enzymatic activity and protein concentration. The incorporation of ^{14}C was determined by liquid scintillation counting using a Packard Tri-Carb counter, Model 3330.

Incorporation of [^{14}C]oADP into the Individual Subunits. Modification of isocitrate dehydrogenase by [^{14}C]oADP and subsequent separation of the modified enzyme from excess reagent were carried out as described. Urea and dithiothreitol were added to the protein samples in final concentrations of 6 M and 4 mM, respectively. The mixtures were then subjected to isoelectric focusing according to a procedure described by Ramachandran & Colman (1980). The gels were stained and destained according to a procedure outlined by Bio-Rad Laboratories, as described by Hayman & Colman (1982). The gels were scanned at 630 nm with a Gilford Model 2410S linear transport attachment to a Model 240 spectrophotometer. For determination of the radioactivity incorporated into the individual protein bands, the gels were cut into 1.4-mm slices. The slices were incubated for 12 h with 0.2 mL of H_2O and 1 mL of Protosol tissue solubilizer at 50–55 °C and counted after addition of 5 mL of Econofluor.

Identification of the Amino Acid Modified by oADP. Isocitrate dehydrogenase was modified with oADP and reduced with $NaBH_4$ as described above. The enzyme was separated from excess reagent and the Pipes buffer system by centrifugation through two consecutive Sephadex G 50-80

[1] Abbreviations: oADP, 2′,3′-dialdehyde derivative of ADP; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); oATP, 2′,3′-dialdehyde derivative of ATP; ACS, aqueous counting scintillant; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

minicolumns (Penefsky, 1979) which had been equilibrated with 25 mM NH_4HCO_3, pH 7.8. For identification of the modified amino acid, the labeled enzyme was subjected to either enzymatic digestion or acid hydrolysis.

Enzymatic digestion of [^{14}C]oADP-labeled isocitrate dehydrogenase was carried out with Pronase (10%) at 25 °C for 14 h, followed by incubation with carboxypeptidase A (10%), carboxypeptidase B (10%), and aminopeptidase M (10%) at 37 °C for 9 h. The digest was repeatedly dissolved in water and evaporated to dryness to remove salts. The sample was analyzed either by application to an amino acid analyzer or by thin-layer electrophoresis.

For identification of the modified amino acid residue after acid hydrolysis, the oADP-labeled isocitrate dehydrogenase was reduced with NaB^3H_4 to introduce the radioactive label. After the filtration step described above, the sample was repeatedly dissolved in water and evaporated to dryness to remove excess salt and then hydrolyzed with 6 N HCl for 20 h at 110 °C. The sample was then analyzed by application to an amino acid analyzer and by thin-layer electrophoresis.

Thin-layer electrophoresis was carried out on cellulose thin-layer sheets with pyridine acetate, pH 6.5 (pyridine/acetic acid/water, 25:1:250), for 90 min at 400 V/20 cm. The dried plates were sprayed with ninhydrin to visualize the amino acids and possible derivatives. ^{14}C or ^{3}H was located on the plates by scanning with a Berthold Radio Scanner LB 2745.

Amino acid analysis was carried out with a Beckman Model 120C amino acid analyzer as described by Likos & Colman (1981). Fractions of the effluent were collected after passage through the photometer at 1.0-min (1.7 mL) intervals. A 1.0-mL aliquot of each fraction was counted in a liquid scintillation counter after addition of 0.2 mL of concentrated HCl and 10 mL of ACS scintillation fluid.

Authentic [^{14}C]Lys-oADP, prepared as described by Easterbrook-Smith et al. (1976), eluted at 78 min. The acid hydrolysis products were prepared by acid hydrolysis of Lys-oADP, as well as by the synthetic method described by Dallocchio et al. (1976). Two lysine derivatives can possibly be obtained after acid hydrolysis of Lys-oADP because the amino group can react with the 2′- or 3′-aldehyde group of oADP, which gives rise to different breakdown products. These derivatives eluted at 191 and 193 min, respectively, well separated between Phe (177 min) and His (203 min). Thin-layer electrophoresis at pH 6.4 also separated these products from all other amino acids.

Calculations of Uncomplexed Ligand Concentrations. Concentrations of the ionic species of ligands were calculated from total concentrations by using a method described by Cohen & Colman (1972), with the association constants for manganese with ligands cited by Ehrlich & Colman (1982b). The pK and association constants for dibasic and tribasic oADP were assumed to be the same as those for ADP.

Results

Inactivation of Isocitrate Dehydrogenase by oADP. Incubation of pig heart isocitrate dehydrogenase with 100 μM oADP resulted in a time-dependent inactivation of the enzyme (Figure 1, oADP). Virtually no change in enzymatic activity was observed when the enzyme was incubated under identical conditions but in the absence of added reagent (Figure 1, control). Biphasic reaction kinetics were observed which could be described in terms of an initial rapid phase, to yield a partially active enzyme of 65% residual activity, followed by a significantly slower phase, to yield totally inactivated enzyme. Throughout this work, the latter phase will be defined as the slow phase; when the contribution of this slow phase is subtracted from the initial phase of inactivation, the remainder is defined as the fast phase (Figure 1, inset).

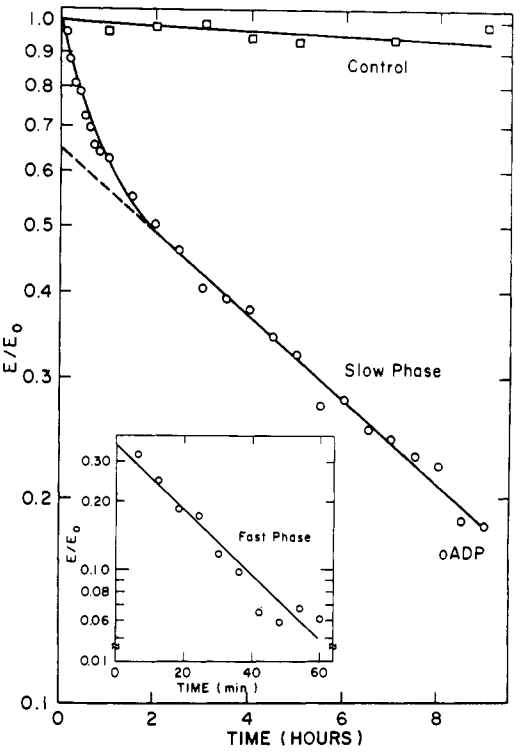

FIGURE 1: Reaction of oADP with isocitrate dehydrogenase. Isocitrate dehydrogenase (0.25 mg/mL) was incubated with oADP (0.1 mM) at 25 °C, in the presence of $MnSO_4$ (2.0 mM), glycerol (20%), and Pipes buffer (50 mM, pH 7.0). At the indicated times, aliquots were removed and assayed for enzymatic activity. As a control, the enzyme was incubated in the absence of oADP but under otherwise identical conditions. The inset represents a replot of the data points obtained during the first 60 min of reaction time with oADP after the contribution of the slow phase of inactivation was subtracted. The contribution of the slow phase at a given time was taken from the linear extrapolation of the slow phase of the reaction. The rate constants obtained from this figure are 0.0024 min^{-1} for the slow phase and 0.032 min^{-1} for the fast phase of inactivation.

One possible explanation for the biphasic nature of the reaction of isocitrate dehydrogenase with oADP may have been reagent decomposition through β-elimination reactions (Khym & Cohn, 1961). However, such instability of the analogue was ruled out in separate experiments in which the purity of [β-^{32}P]oADP was determined by thin-layer electrophoresis (see Experimental Procedures) before and after incubation of the analogue for 6 h in the absence of enzyme but under otherwise standard reaction conditions. After this incubation time, which was in all experiments more than sufficient to have completed the fast phase of the inactivation reaction, no breakdown of [β-^{32}P]oADP to [^{32}P]pyrophosphate and the 4′,5′-unsaturated adenosine derivative was apparent.

For further characterization of the reaction of oADP with isocitrate dehydrogenase, the dependence of the rate of inactivation on the concentration of oADP was determined for the fast and the slow phase. For both phases, a nonlinear dependence of the rate constant on the reagent concentration was observed. These data indicate formation of a reversible complex between oADP and isocitrate dehydrogenase prior to the irreversible step, as is expected for an affinity label (Meloche, 1967). This behavior can be expressed as

$$E + oADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}oADP \xrightarrow{k_2} E_{inact} \qquad (1)$$

where E represents the free enzyme, E–oADP the reversible

Table I: Kinetic Parameters for the Inactivation of Isocitrate Dehydrogenase by oADP[a]

inactivation phase	total [Mn^{2+}] (mM)	$k_2^{oADP(total)}$ (min^{-1})	$K_i^{oADP(total)}$ [b] (μM)	$K_i^{oADP(free)}$ [c] (μM)
slow	0	0.0153	250	
slow	0.2	0.0104	168	25.6
slow	0.3	0.0127	290	28.4
slow	2.0	0.0230	790	15.1
fast	0	0.0920	250	
fast	0.2	0.0860	159	24.7
fast	0.3	0.0870	200	22.0
fast	2.0	0.380	1000	21.7
				22.9 ± 4.6[d]

[a] Isocitrate dehydrogenase (0.25 mg/mL) was inactivated at 25 °C with varying concentrations of oADP in the presence of Pipes buffer (50 mM, pH 7.0) and glycerol (20%), containing EDTA (0.5 mM) or the indicated concentrations of $MnSO_4$. The rates of inactivation by oADP were determined as shown in Figure 1. All kinetic parameters shown in this table were calculated from a least-squares fit of the data to eq 2. [b] $K_i^{oADP(total)}$ represents the inhibition constant based on the total concentrations of oADP present in the inactivation reactions. [c] Only the concentrations of nonchelated oADP present in the reactions were considered for each experiment to obtain $K_i^{oADP(free)}$. These concentrations were calculated from the total concentrations of oADP and $MnSO_4$ as described under Experimental Procedures. [d] Average.

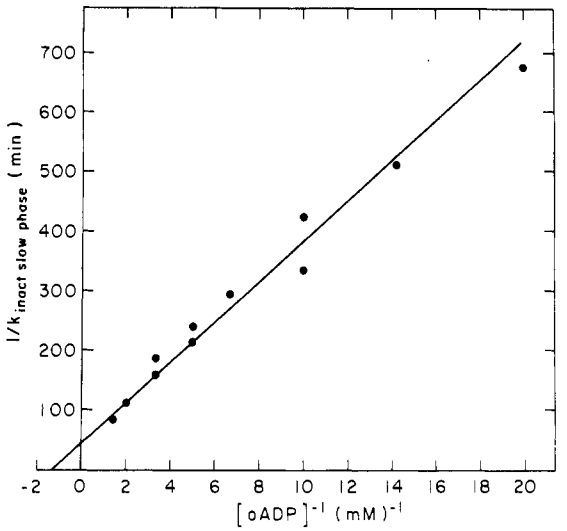

FIGURE 2: Dependence of the slow phase of inactivation on the concentration of oADP. Isocitrate dehydrogenase (0.25 mg/mL) was incubated with varying concentrations of oADP (0.05–0.7 mM). The reaction was carried out at 25 °C in Pipes buffer (50 mM, pH 7.0) containing glycerol (20%) and $MnSO_4$ (2.0 mM). At 30-min intervals and up to 9 h of reaction time, aliquots were withdrawn and assayed for enzymatic activity. The double-reciprocal plot in this figure was then generated from the obtained rates of inactivation of the slow phase and the respective concentrations of oADP employed. The intercepts were calculated by a least-squares fit of the data.

enzyme–oADP complex, and E_{inact} the inactivated enzyme. The observed rate constant is described by

$$k_{obsd} = \frac{k_2}{K_i/[oADP] + 1} \quad (2)$$

where $K_i = (k_{-1} + k_2)/k_1$ and represents that concentration of oADP giving the half-maximal inactivation rate and k_2 represents the maximum rate of inactivation at saturating concentrations of oADP. Figure 2 shows the determination of these kinetic parameters for the slow phase of inactivation by oADP at pH 7.0 and in the presence of 2.0 mM $MnSO_4$. From this double-reciprocal plot of k_{obsd} and [oADP], K_i = 790 μM in terms of total [oADP] and k_2 = 0.023 min^{-1} were obtained.

High-affinity binding of ADP to isocitrate dehydrogenase has been shown to require the presence of free Mn^{2+} ions (Ehrlich & Colman, 1981); in the absence of this divalent metal ion, the activator binds rather weakly to the enzyme. It was therefore of interest to determine the effect of Mn^{2+} ions on the inactivation of isocitrate dehydrogenase by oADP by measuring the dissociation constants for the ADP analogue in the absence and in the presence of 0.2, 0.3, or 2.0 mM $MnSO_4$. As a result of increasing concentrations of metal ions, the K_i values for oADP increased in terms of the total concentration of the analogue; however, on the basis of the concentration of free, nonchelated oADP in the presence of free Mn^{2+}, the values remained relatively unchanged (Table I). The dissociation constants for free oADP at 0.2, 0.3, and 2.0 mM $MnSO_4$ were virtually the same for the two phases of the reaction with an average K_i of 22.9 ± 4.6 μM. These results suggest that inactivation of isocitrate dehydrogenase by oADP may involve tight binding of free oADP to the enzyme in the presence of free Mn^{2+}, in analogy to the metal-dependent high-affinity binding of free ADP. Inactivation of the enzyme by oADP was also observed in the absence of Mn^{2+} ions, but this was associated with a 10-fold higher K_i for free oADP, which is consistent with the weaker binding of ADP to isocitrate dehydrogenase in the absence of metal ions.

The results in Table I also enabled a comparison of the two phases of the inactivation reaction with oADP in terms of their kinetic parameters. Under a given set of conditions, the values for k_2 of the fast phase were 6–16-fold greater than the rate constants for the slow phase while the dissociation constants were almost indistinguishable. This suggests that despite the biphasic nature of the reaction with oADP, the same binding site for oADP may be involved in both phases.

At pH 6.1 and in the presence of 2.0 mM $MnSO_4$, the dissociation constant for free ADP is approximately 2.5 μM (Ehrlich & Colman, 1981). The inhibition constant for oADP determined under identical conditions proved to be strikingly similar; values of 4.2 and 3.5 μM in terms of free oADP were obtained for the fast and slow phases, respectively. In terms of total oADP, the constants were 75.2 μM for the fast phase and 63.8 μM for the slow phase. The k_2 values obtained in these experiments were 0.059 min^{-1} for the fast phase and 0.0054 min^{-1} for the slow phase. At pH 6.1, these rate constants for the reaction of oADP with isocitrate dehydrogenase are several times lower than the rate constants at pH 7.0 (Table I), which is consistent with an inactivation mechanism that involves the typical reaction of an aldehyde with deprotonated forms of amines.

Effect of Added Ligands on the Inactivation of Isocitrate Dehydrogenase by oADP. The effect of added ligands on the reaction of oADP with isocitrate dehydrogenase was investigated separately for the two phases. For determination of its effect on the fast phase of inactivation, each ligand was added simultaneously with oADP to the modification reaction. In the presence of Mn^{2+}, addition of ADP to the inactivation

Table II: Effects of Ligands on the Rate of Inactivation of Isocitrate Dehydrogenase by oADP[a]

ligand	concn (mM)	$k_{obsd} \times 10^{-3}$ [b] + ligand (min^{-1})	oADP(free)[c] (μM)	$k_{calcd} \times 10^{-3}$ [d] − ligand (min^{-1})	(k_{obsd}/k_{calcd}) × 100	K_D(ligand)[e]
			(A) Effects on the Fast Phase			
none		63.0	5.8	63.0	100	
ADP	1.0	9.6	11.7	104.0	9.2	3.9 μM ADP(free)
ADP	2.0	7.9	38.6	190.0	4.2	6.0 μM ADP(free)
isocitrate	1.0	4.0	8.4	83.0	4.8	0.8 μM isocitrate(2−)
isocitrate	3.0	4.5	16.1	127.0	3.6	2.0 μM isocitrate(2−)
isocitrate + ADP	1.0 + 1.0	4.6	18.2	136.0	3.4	
NAD	1.0	63.0	5.8	63.0	100	
NADH	0.1	17.9	5.8	63.0	28.5	
NADH	0.3	15.9	5.8	63.0	25.4	
ATP	1.5	35.0	22.4	151.0	23.2	
ATP[f]	2.0	43.0	48.6	205.0	20.8	
			(B) Effects on the Slow Phase			
none		3.70	5.8	3.70	100	
ADP	1.0	1.63	11.7	6.20	26.5	13.9 μM ADP(free)
ADP	2.0	1.37	38.6	11.40	12.0	19.6 μM ADP(free)
isocitrate	1.0	1.52	8.4	4.90	31.1	7.5 μM isocitrate(2−)
isocitrate	3.0	1.14	16.1	7.50	15.2	10.1 μM isocitrate(2−)
isocitrate + ADP	1.0 + 1.0	0.75	18.2	8.10	9.3	
NAD	1.0	3.50	5.8	3.70	95.7	
NADH	0.1	1.71	5.8	3.70	46.5	
NADH	0.3	1.63	5.8	3.70	44.3	
ATP	1.5	2.50	22.4	9.00	27.8	
ATP[f]	2.0	3.20	48.6	12.40	25.5	

[a] Isocitrate dehydrogenase (0.25 mg/mL) was inactivated at 25 °C with oADP (0.2 mM) in the presence of Pipes buffer (50 mM, pH 7.0), glycerol (20%), $MnSO_4$ (2.0 mM), and the indicated ligands. (A) The ligands and oADP were added simultaneously to initiate the reactions. The rate of inactivation for the fast phase under each of the conditions was determined as described under Figure 1. (B) For determination of the effect of ligands on the slow phase on inactivation, incubation with oADP (0.2 mM) was carried out for 1 h prior to the addition of ligands. The concentration of total oADP was maintained at 0.2 mM throughout the experiment. [b] k_{obsd} + ligands corresponds to the experimentally observed rate of inactivation obtained in the presence of oADP and the added ligand. [c] Due to the chelation of Mn^{2+} by several of the ligands, the concentration of nonchelated oADP [oADP(free)], at the fixed concentration of total oADP (0.2 mM), varied in the presence of the ligands as shown. [d] For each of the calculated concentrations of oADP(free), the respective rate constant k_{calcd} − ligand was estimated by using eq 2 given in the text. [e] All K_D values were calculated by using eq 3. [f] The addition of higher concentrations of ATP was not feasible because of a concomitant decrease in the concentration of free Mn^{2+}, which would cause the concentration of free metal ion to be reduced below the K_D for Mn^{2+}.

reaction resulted in a greater than 10-fold reduction in the rate of inactivation of the fast phase (Table IIA), which suggests that oADP and ADP may compete for the same binding site on the enzyme. For such competition, a binding constant for the ligand can be calculated from the observed rate constants according to

$$k_{obsd} = \frac{k_2}{1 + (K_i/[\text{oADP}])(1 + [\text{L}]/K_D)} \quad (3)$$

where k_{obsd}, K_i, and k_2 are as defined earlier, [L] represents the ligand concentration, and K_D is the binding constant for the ligand. For calculations of the binding constants given in Table II, it was assumed that isocitrate dehydrogenase is inactivated exclusively by the free form of oADP as indicated by the results of Table I. Thus, [oADP] represents the concentration of free oADP in the presence of the individual ligands and K_i = 22.9 μM for free oADP which is the average K_i of the constants in Table I. The rate constants, k_2, were calculated by using eq 2 and determined to be 0.310 and 0.0182 min^{-1} for the fast and the slow phase, respectively. On the basis of the observed rate constants for the fast phase of inactivation in the presence of Mn^{2+} and ADP, binding constants of 3.9 and 6.0 μM were obtained for free ADP (Table IIA). These values are similar to previously obtained binding constants for ADP (Ehrlich & Colman, 1981).

A greater than 20-fold reduction in the rate of the inactivation for the fast phase was obtained when isocitrate was included with Mn^{2+} in the reaction with oADP; addition of ADP simultaneously with isocitrate had no effect on this result. Binding constants for dibasic isocitrate of 0.8 and 2.0 μM were calculated for two different concentrations of isocitrate tested (Table IIA). These values are significantly lower than the dissociation constant of 15 μM reported for dibasic isocitrate at pH 7.0 (Ehrlich & Colman, 1982b), which may suggest that oADP facilitates the binding of isocitrate.

Modification by oADP of the binding site for NAD does not appear to contribute toward the inactivation of isocitrate dehydrogenase by the ADP analogue because addition of NAD to the modification reaction was without effect (Table IIA). NADH and ATP, on the other hand, provided significant protection for the enzyme, but the effects on the rate of inactivation plateaued at reductions of 75–80% of the rate constant of the fast phase (Table IIA).

In the presence of several of the effectors, the rate of inactivation of the enzyme during the fast phase was decreased to such an extent that a differentiation between the fast phase and the slow phase, in order to determine the rate constants, became virtually impossible. Thus, for determination of the effect of added ligands on the slow phase of the reaction, isocitrate dehydrogenase was incubated with oADP for 1 h prior to the addition of each ligand. After the 1-h inactivation time, the slow phase of the reaction predominated. The results obtained by this method for the effect of added ligands on the slow phase of inactivation at pH 7.0 are given in Table IIB.

The calculated binding constants for free ADP and free dibasic isocitrate proved to be severalfold higher for this phase

of the reaction; NADH and ATP were slightly less effective in protecting the enzyme during the slow phase whereas addition of NAD remained without effect. Quantitatively the ligands were less effective for the slow phase of the reaction, which suggests that binding affinities for all of the ligands may have been altered by the modification of isocitrate dehydrogenase with oADP. However, the absence of qualitative differences between protection patterns for the two phases suggests that inactivation of the enzyme may be due to modification of one type of binding site for oADP.

In additional experiments, the effects of added ligands on the inactivation by oADP were determined in the absence of free metal ions. At pH 7.0, this change in modification conditions resulted in dramatic changes in the protection pattern observed: addition of ATP (1.0 mM) or NAD (1.0 mM) had no effect on the inactivation rates while ADP (1.0 mM) or NADH (0.3 mM) decreased the rate constants merely 2-fold.

The effects of added ligands on the inactivation of isocitrate dehydrogenase by oADP were also determined at pH 6.1. For ADP, ATP, NADH, and isocitrate, the results were virtually identical with the results obtained at pH 7.0, but not so for NAD. At pH 6.1, NAD (1.0 mM) decreased the rate of inactivation by more than 10-fold, whereas the same concentration of this substrate had no effect on the reaction at pH 7.0. This differential effect of NAD for the two pH values was unaffected by Mn^{2+} ions. The concentrations of NAD required for a half-maximal effect on the fast and slow phases at pH 6.1 were 490 and 240 μM, respectively. These values are several times greater than the dissociation constant of 55 μM determined for NAD at the same pH (Ehrlich & Colman, 1981), which suggests that the protective effect exerted by NAD on the inactivation of isocitrate dehydrogenase by oADP at pH 6.1 does not involve binding at the high-affinity coenzyme site which is involved in catalysis.

Effect of oADP on the Affinity of Isocitrate Dehydrogenase for Isocitrate. Allosteric activation of NAD-dependent isocitrate dehydrogenase by ADP results in a decrease in the Michaelis constant for isocitrate (Cohen & Colman, 1972). When the activator is added to isocitrate dehydrogenase assays containing nonsaturating concentrations of isocitrate, an increase in the initial velocity is observed. A 3.5-fold increase in the initial reaction velocity of the native enzyme was produced in Pipes buffer (pH 7.0) by addition of 0.5 mM ADP to assays with 0.5 mM isocitrate. This method of measuring the allosteric activation of isocitrate dehydrogenase was also employed with oADP (1.0 mM), but addition of this ADP analogue resulted in virtually no change in the initial reaction velocity. Activation was also absent when oADP was converted to the dialcohol derivative through reduction with $NaBH_4$ before it was added to the assays, which indicates that an intact ribosyl ring of ADP may be essential for the allosteric activation of isocitrate dehydrogenase.

Despite the observation that addition of oADP had no effect on the initial velocities of isocitrate dehydrogenase with nonsaturating concentrations of isocitrate, it was still conceivable that covalent incorporation of the analogue might produce a decrease in the K_m for isocitrate. Isocitrate dehydrogenase was incubated with oADP under standard modification conditions to test for this possibility. At intervals, aliquots were removed and assayed for enzymatic activity at saturating concentrations of isocitrate (20 mM) and at nonsaturating concentrations of substrate (0.5 mM). If modification of the enzyme altered the K_m for isocitrate, then the apparent inactivation rates, as measured at these two substrate concentrations, would not be the same. However, since inactivation

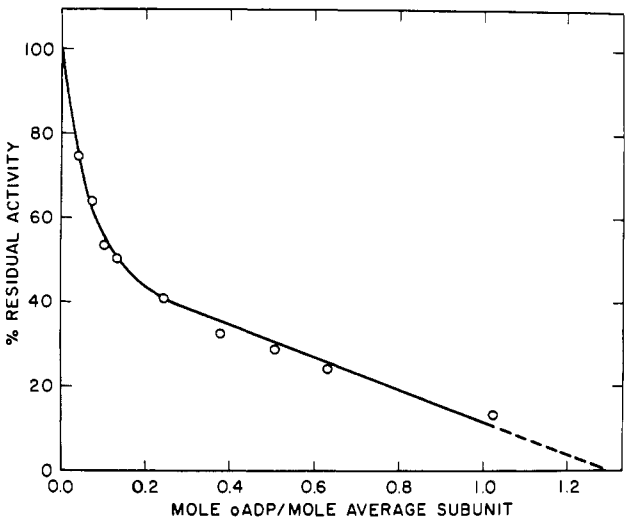

FIGURE 3: Correlation between inactivation of isocitrate dehydrogenase and incorporation of [^{14}C]oADP. Isocitrate dehydrogenase (0.4 g/mL) was incubated at 25 °C with [^{14}C]oADP (0.2 mM at 1 Ci/mol), in the presence of Pipes buffer (50 mM, pH 7.0), glycerol (20%), and $MnSO_4$ (2.0 mM). At intervals, aliquots were withdrawn, separated from excess reagent, and assayed for enzymatic activity and incorporation of [^{14}C]oADP as described under Experimental Procedures.

rates with oADP were found to be virtually identical (data not shown), it appears unlikely that the K_m for isocitrate was altered significantly upon modification of the enzyme.

For determination of the effect of modification of isocitrate dehydrogenase on the allosteric activation of the enzyme by ADP, inactivation by oADP was again measured in subsequent assays with nonsaturating concentrations of isocitrate (0.5 mM) but either in the absence or in the presence of ADP (1.0 mM). The observed rate constants for each of the two phases of the inactivation by oADP remained very similar under both conditions (data not shown). This result eliminates the possibility that incorporation of oADP on some subunits influences the effect on ADP on the binding of isocitrate on unmodified subunits.

Stoichiometry of Reaction of Isocitrate Dehydrogenase with [^{14}C]oADP. An estimate of the specificity of an affinity label for a particular binding site can be obtained by determining the stoichiometry of the reaction between the analogue and the enzyme. In an ideal case, the number of moles of analogue incorporated per mole of enzyme should not exceed the number of ligand binding sites. It was therefore of interest to determine the incorporation of [^{14}C]oADP into isocitrate dehydrogenase as a function of the loss of enzymatic activity. The results thus obtained for the modification reaction at pH 7.0 are given in Figure 3. An extrapolated value of approximately 1.3 mol of oADP per mol of average subunit can be obtained when the enzyme is completely inactivated. The correlation between the incorporation of oADP and the loss of enzymatic activity was biphasic, as it was for the kinetics of the inactivation reaction (Figure 1). The possibility that the initial phase of the reaction, which corresponded to very low incorporation values, may have been due to noncovalent interactions was considered. oADP was reduced by $NaBH_4$ prior to incubation with the enzyme to test for this possibility. This treatment converted the two reactive aldehyde groups of oADP to the unreactive alcohols. The dialcohol derivative appeared to compete with oADP in binding to isocitrate dehydrogenase because it partially protected the enzyme from inactivation by oADP. However, when added under standard modification conditions to isocitrate dehydrogenase and incubated with the enzyme for 1 h, the dialcohol derivative (1.0 mM) had no

Table III: Subunit Labeling of Isocitrate Dehydrogenase by [^{14}C]oADP[a]

% residual activity	mol of oADP/mol of average subunit	% of total radioactivity[b]			% of total protein[c]		
		α	β	γ	α	β	γ
100.0					48.7	25.3	26.0
54.5	0.086	52.3	28.5	14.0	53.1	24.3	22.7
30.6	0.316	54.3	28.9	13.0	57.8	23.6	18.6

[a] Isocitrate dehydrogenase (0.5 mg/mL) was incubated at 25 °C with [^{14}C]oADP (0.2 mM at 3 Ci/mol) in the presence of Pipes buffer (50 mM, pH 7.0), glycerol (20%), and $MnSO_4$ (2.0 mM). After 1 and 4 h, some aliquots were assayed for activity while others were treated with $NaBH_4$, separated from excess reagent, and subjected to isoelectric focusing as described under Experimental Procedures. [b] Protein bands corresponding to the individual subunits of isocitrate dehydrogenase were cut from the gels. The incorporation of radioactivity into the individual slices, after solubilization of the radioactive label, was determined by liquid scintillation counting (see Experimental Procedures). [c] The distribution of protein between the individual groups of subunits was determined from the optical density of the respective protein bands at 630 nm.

effect on subsequent assays for enzymatic activity. The data suggest that reversible binding of oADP to the enzyme may be insufficient to cause inactivation; covalent incorporation is required.

Alternatively, one may propose that the covalent bond between oADP and isocitrate dehydrogenase is readily reversible and that some of the radioactive label is lost during the 7 min of filtration time. This would account for the low incorporation values at the earlier times. It should be noted, however, that the loss of enzymatic activity was determined after the filtration step, which would indicate that the loss of enzymatic activity is not as readily reversed. Formation of a Schiff base between oADP and the ε-amino group of a lysine residue on isocitrate dehydrogenase is one possible reaction mechanism that leads to a reversible product. A Schiff base is relatively unstable and must generally be converted to a stable amine by reduction with $NaBH_4$ or $NaCNBH_3$. However, when reduction of the modified isocitrate dehydrogenase was carried out with a 150-fold molar excess of $NaBH_4$ over oADP prior to the filtration step, the same values for the incorporation of [^{14}C]oADP were obtained as those obtained without reduction.

The stoichiometry of the reaction between oADP and isocitrate dehydrogenase was also determined at pH 6.1 by utilizing the same procedures that were used at pH 7.0. It was postulated that a lowering of the pH might decrease the possibility of nonspecific modification of the enzyme. However, the results of such a correlation between the incorporation and the inactivation of isocitrate dehydrogenase at pH 6.1 were almost identical with those obtained at pH 7.0; labeling at pH 6.1, for any given residual activity, was less than 0.05 mol of oADP/mol of average subunit below the incorporation value at pH 7.0.

Labeling of the Individual Subunits of Isocitrate Dehydrogenase by [^{14}C]oADP. The identity of the ADP binding subunit(s) of isocitrate dehydrogenase was investigated by determining the incorporation of radioactive oADP into the three types of subunits. In these experiments, isocitrate dehydrogenase was modified by [^{14}C]oADP under standard modification conditions at pH 7.0, followed by separation of the individual subunits by isoelectric focusing on polyacrylamide gels. As a control, isocitrate dehydrogenase was treated under identical conditions but in the absence of oADP. The results for the distribution of protein bands and radioactive label are shown in Figure 4. The native enzyme focused into three distinct groups of protein bands corresponding to the α, β, and γ subunits (Figure 4A). The ranges of the isoelectric points of the groups were previously reported to be 5.6–5.8, 6.4–6.7, and 6.9–7.5 for the multiple bands of the α, β, and γ subunits, respectively (Ramachandran & Colman, 1980). Upon modification with [^{14}C]oADP, changes in the distribution of protein bands were obtained for all types of subunits of isocitrate dehydrogenase (Figure 4B). The overall result

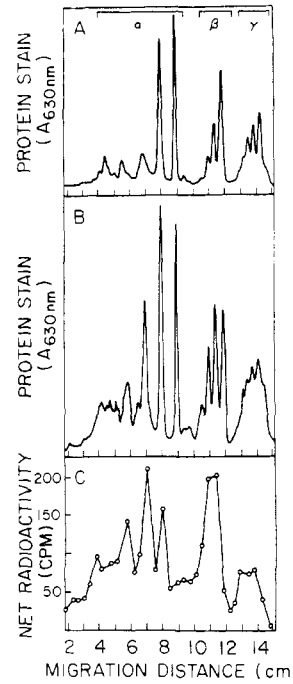

FIGURE 4: Labeling of the individual subunits of isocitrate dehydrogenase by [^{14}C]oADP. Isocitrate dehydrogenase (0.5 mg/mL) was incubated at 25 °C with or without [^{14}C]oADP (0.2 mM at 3 Ci/mol) in the presence of Pipes buffer (50 mM, pH 7.0), glycerol (20%), and $MnSO_4$ (2.0 mM). After 4 h, aliquots were assayed for enzymatic activity. The remainder of the samples were treated with three additions of $NaBH_4$ (final concentration 30 mM) in 5-min intervals. The protein was separated from excess reagent and subjected to isoelectric focusing as described under Experimental Procedures. (A) Protein staining pattern of native isocitrate dehydrogenase which had been incubated in the absence of oADP. (B) Protein staining pattern of isocitrate dehydrogenase modified by 0.31 mol of oADP/mol of average subunit. (C) Distribution of radioactivity in the individual protein bands. The identical gel was used to obtain (B) and (C).

was a shift of protein within one group of subunits, from bands at a higher p*I* to bands at a lower p*I* (i.e., a shift toward a smaller migration distance).

The incorporation of [^{14}C]oADP into the protein bands (Figure 4C) was found to correlate well with the changes in protein distribution in that the protein bands increasing in intensity the most upon modification also contained the largest amounts of radioactive label, whereas the protein bands which decreased relatively were associated with much lower incorporation of [^{14}C]oADP.

Table III shows a quantitation of the distribution of protein and radioactivity within the three groups of subunits before and after modification by [^{14}C]oADP. The quantitation was carried out at an earlier and a later time point of modification to ascertain whether the two phases of the reaction with oADP may be due to differences in the reactivity of the analogue with

the three types of subunits. The results shown in Table III for the distribution of protein and radioactivity were the same for either phase of the reaction which eliminates this possible explanation for the biphasic behavior of oADP with isocitrate dehydrogenase. Quantitation of protein of the three types of subunits showed an approximate ratio of 2α, 1β, and 1γ for tne native enzyme and only minor changes in this ratio upon modification. The corresponding approximate ratio for the incorporation of [^{14}C]oADP into the modified enzyme was 2α, 1β, and 0.5γ, showing significant labeling of all three subunits by the ADP analogue. Modification of the three types of subunits of isocitrate dehydrogenase by oADP suggests that the structurally distinct subunits may all contain a similar allosteric site for ADP.

Identification of the Amino Acid Residue Modified by oADP. Reaction of dialdehyde derivatives with the ϵ-amino group of lysine residues has previously been shown to lead to the formation of Schiff base products which can subsequently be stabilized by reduction with $NaBH_4$ or $NaCNBH_3$ (Easterbrook-Smith et al., 1976; Dallocchio et al., 1976). In these studies, radioactively labeled enzyme was subjected to enzymatic digestion or acid hydrolysis; the resulting radioactive products were then compared to the authentic lysine derivatives by thin-layer chromatography. This approach was also utilized in the present study to allow identification of the modified amino acid residue in isocitrate dehydrogenase; however, instead of thin-layer chromatography, either thin-layer electrophoresis or separation by an amino acid analyzer was employed as the analytical technique for the comparisons (see Experimental Procedures). Despite reduction of oADP-labeled enzyme by a 150-fold molar excess of $NaBH_4$ over oADP, no lysine derivatives expected from a Schiff base mechanism were detected by either method. In fact, after thin-layer electrophoresis of the enzymatic digest at pH 6.4, almost all of the radioactivity was associated with the neutral amino acids near the origin, even though a negatively charged product was expected. Furthermore, reduction of oADP-labeled isocitrate dehydrogenase by NaB^3H_4, at earlier or later inactivation times, provided no more than the basal radioactivity that was obtained when the unmodified control enzyme was reduced with NaB^3H_4. These results provide evidence against a Schiff base product for the inactivation of isocitrate dehydrogenase by oADP and suggest a reaction mechanism which renders both aldehyde groups unavailable for reduction.

Reaction of oADP with the free N terminals of the three subunits of isocitrate dehydrogenase could possibly lead to inactivation of the enzyme. As a test for this mechanism, the N terminals of isocitrate dehydrogenase were quantitated, by utilizing a dansylation procedure described by Gros & Labouesse (1969). The results were the same for native and modified enzyme (data not shown), which argues against the reaction of oADP with these α-amino groups.

In a recent study on the inactivation of mitochondrial adenosinetriphosphatase by oATP (Lowe et al., 1979), it was proposed that an elimination reaction occurred with enzyme-bound oATP which liberated the triphosphate group and formed a very stable conjugated Schiff base. The enzyme was modified with [^{14}C]oADP and [^{32}P]oADP under standard conditions to test for β elimination of the pyrophosphate group from oADP bound to isocitrate dehydrogenase. At 66% inactivation of the enzyme, 0.41 mol of [^{14}C]oADP/mol of average subunit was incorporated, whereas only 0.014 mol of [^{32}P]oADP/mol of average subunit was bound to isocitrate dehydrogenase. This dramatic difference between the values obtained with [^{14}C]oADP and [^{32}P]oADP was also seen at the earlier inactivation times and proved to be unaffected by reduction of the modified enzyme with $NaBH_4$. In separate experiments, the release of [^{32}P]pyrophosphate was monitored during the modification reaction to ascertain whether a conversion of oADP to the elimination product might be responsible for the biphasic reaction kinetics, but no significant increase in the release of [^{32}P]pyrophosphate, beyond that released from enzyme-bound oADP, was found. These results show that the rapid elimination of the pyrophosphoryl group involves only enzyme-bound oADP. The elimination reaction could lead to the formation of a stable conjugated Schiff base which may not require reduction by $NaBH_4$ to stabilize the analogue on the enzyme. However, this type of aldehyde fixation (Monsan et al., 1975) of the 3′-aldehyde group should have allowed incorporation of ^{3}H at least into the unreacted 2′-aldehyde group when oADP-labeled isocitrate dehydrogenase was reduced by NaB^3H_4.

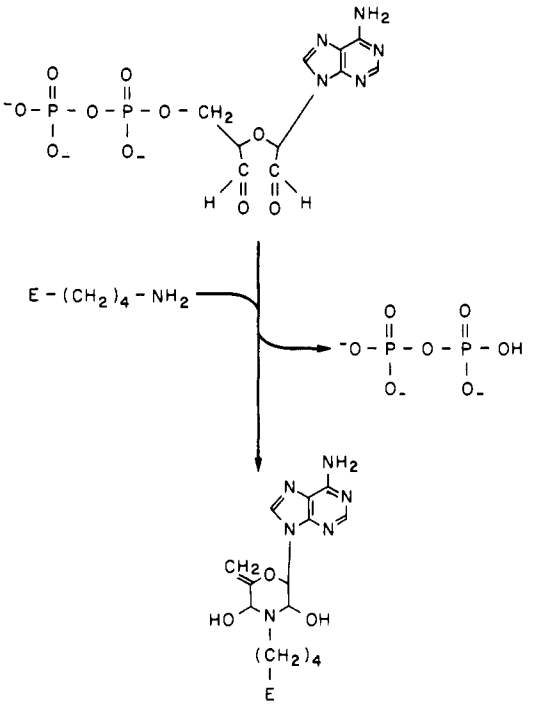

FIGURE 5: Proposed reaction mechanism for the inactivation of isocitrate dehydrogenase by oADP at pH 7.0.

One possible reaction product that involves both aldehyde groups of oADP is a dihydroxymorpholino derivative. Such a product was suggested by Gregory & Kaiser (1979) for the inactivation of phosphofructokinase by oATP. The derivative is relatively stable and would account for the fact that reduction by $NaBH_4$ was not necessary to stabilize [^{14}C]oADP on isocitrate dehydrogenase for the determination of stoichiometry and subunit labeling.

The mechanism for the reaction of oADP with isocitrate dehydrogenase that is, therefore, most consistent with the obtained data involves the formation of a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative with lysine as is shown in Figure 5. This unsaturated dihydroxymorpholino derivative would be relatively stable in the absence of reduction. If one assumes that this derivative is a structural analogue of Mes [2-(*N*-morpholino)ethanesulfonic acid], which has a pK_a of 6.15 (Good et al., 1966), this mechanism would also be consistent with the formation of a neutral product at pH 6.4. In fact, the difference in the pK_a for lysine and the dihydroxymorpholino derivative may be sufficient to explain the shift of subunit bands to a slightly lower p*I* upon modification of isocitrate dehydrogenase with oADP (Figure 4). Despite the

relative stability of the obtained product, which allowed isoelectric focusing of oADP-labeled enzyme and the determination of stoichiometry without prior reduction, attempts at separating the presumed derivative from all other amino acids were unsuccessful.

Discussion

Affinity labeling of NAD-dependent isocitrate dehydrogenase by oADP resulted in preferential modification of the allosteric binding site for ADP as indicated by the observation of saturation kinetics, protection by the natural ligand, similarities in binding affinities for ADP and oADP, the effect of Mn^{2+} ions, and the stoichiometric incorporation of this structural analogue of ADP into the enzyme. In addition to the protection by ADP, which was consistent with its directly determined binding constant, isocitrate dehydrogenase was almost totally protected by isocitrate. These results are consistent with the previously indicated interactions between the binding sites for ADP and isocitrate. ADP has been shown to decrease the K_m (Cohen & Colman, 1972) as well as the K_D (Ehrlich & Colman, 1981) for isocitrate. The effector also protected the substrate binding site of isocitrate dehydrogenase from modification by 2,3-butanedione (Hayman & Colman, 1978) and 3-bromo-2-ketoglutarate (Bednar et al., 1982a). These previous results indicated conformational changes in the catalytic site upon binding of ADP to the allosteric site. It is conceivable that such communication between the sites is reciprocal, which would account for the protection of isocitrate dehydrogenase afforded by isocitrate from inactivation by oADP.

Direct binding studies have shown that metal-dependent binding of ADP to a high-affinity binding site on isocitrate dehydrogenase requires the free, nonchelated form of the activator. Very similar behavior was also observed for the inactivation of isocitrate dehydrogenase by oADP when determinations of the K_i values for oADP were carried out with increasing concentrations of Mn^{2+} ions. The results showed increasing dissociation constants for oADP in terms of the total concentration of the analogue but virtually unchanged values in terms of free oADP, which suggests that in the presence of Mn^{2+} ions, inactivation of isocitrate dehydrogenase by free oADP is caused by modification of the metal-dependent high-affinity ADP binding site.

The inactivation of isocitrate dehydrogenase by oADP proceeded in two, kinetically distinct phases: an initially rapid phase was followed by a significantly slower phase. The similarity in the subunit distribution of the affinity label for the earlier and later time points of the reaction indicated that differences in the reactivity of the three types of subunits with oADP cannot be responsible for the two phases. Analysis of the two phases of the reaction in terms of the concentration dependence of the reaction, the effect of Mn^{2+} ions, and the protection pattern by substrates and effectors provided virtually identical results, suggesting that both phases of the inactivation correspond to the modification of one type of binding site for oADP.

The two rates of reaction of oADP with isocitrate dehydrogenase may represent rapid reaction of the analogue with the first of four subunits followed by subsequently slower reaction with the remaining subunits. A decreasing rate of inactivation was also seen for the substrate affinity label 3,4-didehydro-2-ketoglutarate (Bednar et al., 1982b). The change in the rate of reaction may be the result of conformational changes due to the initial reaction. This may, in fact, be another reflection of the negatively cooperative interactions suggested by the tight binding of natural ligands to only half of the subunits of isocitrate dehydrogenase (Ehrlich & Colman, 1981).

Modification of isocitrate dehydrogenase by oADP occurred in two distinct phases: affinity labeling of the first of four average subunits had a dramatic effect on the V_{max} of the enzyme and caused 50% inactivation; subsequent labeling of the remaining three subunits had proportionally less effect on the isocitrate dehydrogenase activity. However, labeling of about one site per average subunit was eventually obtained with the second phase of modification, corresponding to total inactivation. This result indicated the presence of one binding site for oADP (and potentially ADP) for every subunit of isocitrate dehydrogenase. Since binding studies have provided evidence for tight binding of ADP at only one binding site per two average subunits (Ehrlich & Colman, 1981), the stoichiometry of labeling of oADP implies that the negative cooperativity may be more extreme in the case of the binding of the natural activator, ADP, than in the binding of its analogue, oADP.

Labeling of all subunits was observed at different stages of the reaction, indicating that it may not matter which subunit is initially attacked. These results support the postulate that the structurally distinct subunits of isocitrate dehydrogenase may be functionally equivalent. Evidence for the latter assumption was obtained in previous studies in which catalytic activity was observed with the separated α and β subunits of isocitrate dehydrogenase (Hayman & Colman, 1982; Ehrlich & Colman, 1982a). Furthermore, modification of isocitrate dehydrogenase with reagents directed toward the binding site for isocitrate resulted in labeling of all three types of subunits (Bednar & Colman, 1982). Since ADP, ATP, NAD, NADH, NADPH, Mn^{2+}, and isocitrate bind to one site per two subunits (Ehrlich & Colman, 1981, 1982b), it may be that negative cooperative interactions exist among the subunits upon binding of all these ligands. One might see the tetrameric enzyme as a dimer of dimers with a distinct conformation for each dimer and a "flip-flop" between the two conformations as an obligatory step in the enzyme mechanism of isocitrate dehydrogenase. On the basis of this model, a given ligand, like ADP, may bind preferentially to one of the two major forms of the dimers, being obligatorily released before the alternate conformation can be assumed. Covalent incorporation of any ligand analogue would therefore prevent the dimers from alternating in conformation and cause inactivation of the enzyme. Thus, inactivation of isocitrate dehydrogenase by the ADP analogue oADP may be understandable even though oADP appears to react at the enzyme's activator site.

In order to obtain structural information on the binding site for ADP, it was of interest to identify which amino acid residue(s) in isocitrate dehydrogenase became modified by oADP. A dialdehyde derivative of a nucleotide, such as oADP, is generally assumed to react with the ϵ-amino group of a lysine residue on a given enzyme to form a reversible Schiff base product. Results which are consistent with this mode of action were obtained in studies by Easterbrook-Smith et al. (1976) and Dallocchio et al. (1976), who employed the affinity labels oATP and oNADP, respectively. The methodologies that were described by these investigators for the identification of the modified lysyl residues were employed and expanded upon in the present study, but the results showed a definite absence of any lysyl derivatives expected from this Schiff base mechanism.

In recent years, two alternative reaction products between dialdehyde derivatives and lysine residues have been proposed for which the reduction with $NaBH_4$ to stabilize the en-

zyme–analogue complex appeared unnecessary. Elimination of the triphosphate group of oATP bound to mitochondrial adenosinetriphosphatase (Lowe et al., 1979) was initially proposed to lead to the formation of a stable conjugated Schiff base with lysine. Subsequently, while this paper was in preparation, an alternate mechanism was suggested for the inactivation of this adenosinetriphosphatase by oATP which involved the formation of a morpholino derivative with lysine (Lowe & Beechey, 1982). Formation of a stable dihydroxymorpholino derivative of lysine was previously suggested for the reaction of oATP with phosphofructokinase (Gregory & Kaiser, 1979) and phosphorylase kinase (King & Carlson, 1981). The results obtained in the present study of the inactivation of isocitrate dehydrogenase by oADP also provided evidence for an elimination reaction, which led to a loss of the pyrophosphate group from the enzyme-bound oADP and suggested that the final product is a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative.

Registry No. oADP, 64060-84-0; ADP, 58-64-0; ATP, 56-65-5; NADH, 58-68-4; isocitric acid, 320-77-4; L-lysine, 56-87-1; isocitrate dehydrogenase, 9001-58-5; Lys-oADP, 84560-16-7.

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Chemical Modification of L-Lactate 2-Monooxygenase with Fluorodinitrobenzene: Evidence for Two Essential Histidine Residues†

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ABSTRACT: The modification of L-lactate 2-monooxygenase (lactate oxidase) with radioactively labeled fluorodinitrobenzene in 50 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, pH 6.0, resulted in complete inactivation with the incorporation of 1 mol of dinitrophenyl per catalytic site. Analysis of acid hydrolysates using high-pressure liquid chromatography and an amino acid analyzer showed that N^{im}-(dinitrophenyl)histidine accounted for 95% of the incorporated label. Approximately 50% of the flavin in the modified enzyme was reduced by lactate, formed a sulfite complex, and underwent a photochemical reaction with oxalate. These results suggest that each active site in lactate oxidase contains two essential histidine residues that are modified in a mutually exclusive manner.

L-Lactate 2-monooxygenase (lactate oxidase) (EC 1.13.12.4) from mycobacteria catalyzes the oxidative decarboxylation of L-lactate and other L α-hydroxy acids according to the equation:

$$R\cdot CHOH\cdot COOH + O_2 \rightarrow R\cdot COOH + H_2O + CO_2$$

This FMN-containing flavoenzyme has been classified as a dehydrogenase/oxidase (Massey & Hemmerich, 1980). Ex-

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