Identification of the Subunit and Important Target Peptides of Pig Heart NAD-Dependent Isocitrate Dehydrogenase Modified by the Affinity Label Adenosine 5′-*O*-[*S*-(4-Bromo-2,3-dioxobutyl)thiophosphate][†]

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ABSTRACT: Pig heart NAD-dependent isocitrate dehydrogenase is inactivated by adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPS-BDB) with incorporation of 1.78 mol of reagent/mol of average subunit. Complete protection against the inactivation is provided by 20 mM isocitrate + 1 mM Mn^{2+}, and the incorporation is decreased to about 1.3 mol of reagent/mol of average subunit. The addition of NAD, NADH, or Mn^{2+} alone has little effect on the functional changes produced by AMPS-BDB, while ADP gives only partial protection against the inactivation. The ability of ADP to decrease the K_m for isocitrate is not affected by the AMPS-BDB modification of the enzyme. These results indicate that the isocitrate substrate site is the target of AMPS-BDB. The enzyme has three types of subunits with a tetramer having the composition $\alpha_2\beta\gamma$. Here, [2-^{3}H]AMPS-BDB-modified subunits are separated by HPLC on a C_4 reverse-phase column, after the treatment of the modified enzyme with 4 M urea. The predominant radioactivity is distributed in α and γ subunits. However, evidence based on recombination of subunits from modified and unmodified enzymes indicates that only labeling of the α subunit is responsible for inactivation by AMPS-BDB. Subsequently, the separated modified subunits were chemically cleaved by CNBr and then purified by HPLC using a C_{18} column. The labeled peptides were further digested by pepsin, purified by HPLC, and sequenced. These results indicate that R^{88} and R^{98} from the α subunit are the major targets of AMPS-BDB which cause inactivation and that these are at or near the isocitrate site of the enzyme.

The NAD-specific isocitrate dehydrogenase from pig heart is a mitochondrial allosteric enzyme regulated by ADP, which activates the enzyme by lowering the K_m for isocitrate without changing the maximum velocity (*1*). This enzyme is a tetramer with three distinct types of subunits in the ratio 2α:1β:1γ, whose molecular weights are similar (about 40 000) while their isoelectric points are distinctive (*2, 3*). Direct binding experiments have shown that there are two tight binding sites for ADP, isocitrate, Mn^{2+}, NAD, and NADH for a tetramer (*4, 5*). However, the ligand sites have not clearly been associated with particular subunits.

Chemical modification of isocitrate dehydrogenase has implicated several types of amino acid important for catalysis and regulation, including lysine, glutamate, aspartate, cysteine, and arginine (*6*). Asp^{190} derived from the γ subunit was found at or near the ADP site by affinity labeling with the ADP analogue 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (*7*). The cDNA for human NAD-specific isocitrate dehydrogenase α subunit has been isolated, and its deduced amino acid sequence has been compared with others from different species (*8*). The peptide sequence data obtained from the pig heart enzyme has been assembled (*9*) by comparing the sequences of the corresponding subunits reported for the monkey (*10, 11*), bovine (*12, 13*), and human (*8*) enzymes.

Adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPS-BDB)[1] is an ADP analogue synthesized in this laboratory (*14*), which has a reactive bromodioxobutyl group at a position equivalent to that of pyrophosphate. It acts as an affinity label of the active site of rabbit muscle pyruvate kinase (*14*) and functions as an affinity label of the ADP regulatory site of bovine liver glutamate dehydrogenase (*15*).

In this study, we report that AMPS-BDB reacts covalently with NAD-dependent pig heart isocitrate dehydrogenase to produce an inactive, irreversibly modified enzyme. The evidence suggests, surprisingly, that AMPS-BDB reacts at the isocitrate substrate rather than at the allosteric ADP site. This study also describes the isolation of AMPS-BDB-modified subunits and identifies the modified amino acid residues responsible for the inactivation.

EXPERIMENTAL PROCEDURES

Materials. Frozen pig hearts were supplied by Pel Freeze. NAD and the other coenzymes, DL-isocitrate, dithiothreitol,

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[1] Abbreviations: AMPS-BDB, adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TFA, trifluoroacetic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; CNBr, cyanogen bromide; HPLC, high-performance liquid chromatography; TTAB, tetradecylammonium bromide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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PIPES buffer, HEPES buffer, phenylglyoxal, tetradecylammonium bromide (TTAB), methyl-β-cyclodextrin, and Sephadex G-50-80 were purchased from Sigma. $MnSO_4$ and sodium bicarbonate were supplied by Fisher Scientific, and ultrapure ammonium sulfate was supplied by ICN. The 1,4-dibromobutanedione was obtained from Aldrich and was recrystallized from petroleum ether prior to use. 2-[^{3}H]-Adenosine and [^{3}H]$NaBH_4$ in 0.1 N NaOH were obtained from Amersham Life Sciences. Whatman supplied the DE-52 cellulose, and Sigma provided cellulose phosphate.

Enzyme Assays. The activity of NAD-dependent isocitrate dehydrogenase was monitored at 25 °C by the reduction of NAD ($\epsilon_{340\ nm} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) using a Perkin-Elmer spectrophotometer with an expanded scale (0.0−0.1). The assay buffer contained 20 mM DL-isocitrate, 1 mM $MnSO_4$, and 1 mM NAD in 33 mM Tris-acetate, pH 7.2, in a total volume of 1 mL. One enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min at 25 °C, and the specific activity is defined as the number of enzyme activity units per milligram of protein. The Michaelis constants for isocitrate of native and AMPS-BDB-modified enzyme were determined under the standard assay conditions except for the varying concentration of isocitrate in the absence and presence of 1 mM ADP.

Purification of Pig Heart NAD-Dependent Isocitrate Dehydrogenase. NAD-dependent isocitrate dehydrogenase was prepared from frozen pig hearts supplied by Pel Freeze. It was purified by the procedures reported by Ehrlich et al. (*2*), a modification of the method of Ramachandran and Colman (*3*), and was stored at −80 °C. Prior to the modification and protection reactions, the enzyme was dialyzed against 50 mM PIPES, pH 7.0, containing 20% glycerol and 0.2 mM $MnSO_4$.

Synthesis of Adenosine 5′-O-[S-(4-Bromo-2,3-dioxobutyl)-thiophosphate] (AMPS-BDB). The overall synthetic scheme followed the procedure of Vollmer et al. (*14*). In the case of radioactive [2-^{3}H]AMPS-BDB, 1 mCi of [2-^{3}H]adenosine was added to 0.8 mmol of adenosine. The remaining procedures were the same as those in Vollmer et al. (*14*).

Reaction of Pig Heart NAD-Dependent Isocitrate Dehydrogenase with AMPS-BDB in the Absence and Presence of Protecting Ligands. Enzyme (0.10 mg/mL) was preincubated in 50 mM PIPES buffer, pH 7.0, containing 20% glycerol and 0.2 mM $MnSO_4$ at 25 °C for 20 min. To initiate the reaction, the AMPS-BDB in water was added to yield concentrations from 0.1 to 1.0 mM. In the protection studies, various concentrations of substrates and regulatory ligands were added to the preincubation mixture. The control enzyme was subjected to the same procedure in the absence of the compound and ligands. Aliquots were withdrawn during the reaction at various times and assayed spectrophotometrically for isocitrate dehydrogenase activity. Reaction rates were determined from the initial 5 min of reaction.

Incorporation of [2-^{3}H]AMPS-BDB into NAD-Dependent Isocitrate Dehydrogenase. Enzyme (0.10 mg/mL) was incubated with 0.4 mM [2-^{3}H]AMPS-BDB at 25 °C either for 60 min or for 2 h under the conditions described above. At the end of the incubation period, $NaBH_4$ was added to give the final concentration of 10 mM to reduce the carbonyls of AMPS-BDB and thereby stop the reaction. The reaction mixture was maintained on ice for 15 min, and then *N*-ethylmaleimide (NEM, final concentration of 20 mM) was added to block free cysteine SH groups. After 10 min on ice, modified enzymes were separated from the excess reagent by column centrifugation (*16*) using one minicolumn (5 mL) filled with Sephadex G-50-80 equilibrated with 50 mM HEPES, pH 7.0. Subsequently, the enzyme was dialyzed against 0.1% trifluoroacetic acid (TFA) in H_2O. After the overnight dialysis, aliquots were taken to determine the reagent incorporation. The concentration of modified enzyme was determined by the Bio-Rad protein assay based on the method reported in Bradford (*17*) with the unmodified enzyme as the standard. The amount of reagent incorporated was determined from the radioactivity of modified enzyme measured in 5 mL of Liquiscint scintillation cocktail using a Packard Tricarb model 3300 liquid scintillation counter. The specific radioactivity of [2-^{3}H]AMPS-BDB was 1×10^{12} cpm/mol.

Reduction of AMPS-BDB-Modified Enzyme by [^{3}H]-$NaBH_4$. The reduction of the carbonyl groups of AMPS-BDB by [^{3}H]$NaBH_4$ would introduce a radioactive tag to the modified enzyme (*7*). The reduction can be accomplished by adding [^{3}H]$NaBH_4$ to the modified enzyme; 2 mol of tritium should be introduced for each mole of dioxobutyl group of AMPS-BDB. Enzyme (0.5 mg/mL) containing 50 mM PIPES, 20% glycerol, and 0.2 mM $MnSO_4$ was preincubated for 20 min at 25 °C. Then 200 μM AMPS-BDB was added to the reaction mixture. The reaction was followed spectrophotometrically for 70 min, resulting in 45% residual activity. After the addition of 192 mM [^{3}H]$NaBH_4$ in 10 mM NaOH (to yield 10 mM $NaBH_4$ as the final concentration), the reaction mixture was maintained on ice for 15 min in the hood. The samples were transferred to dialysis bags and dialyzed against 1 L of 0.1% TFA buffer with three changes for 20 h to remove free radioactive $NaBH_4$ and reagent. The Bio-Rad protein concentration assay was used to determine the concentration of modified enzyme after the dialysis. The amount of reagent incorporated was determined from the radioactivity of modified enzyme. In the case of "protected" reactions, 1 mM $MnSO_4$ and 20 mM isocitrate were included in the preincubation mixture. The specific radioactivity of [^{3}H]$NaBH_4$ was 0.61×10^{12} cpm/mol of hydrogen. A control enzyme was run under the same conditions.

Subunit Separation of Modified Isocitrate Dehydrogenase Pretreated with 4 M Urea. Enzyme (0.93 mg/mL) was incubated with 0.5 mM [2-^{3}H]AMPS-BDB for 2 h, as described above. After passing through one Sephadex G-50-80 minicolumn equilibrated with 50 mM HEPES, pH 7.0, containing 4 M urea, followed by another minicolumn equilibrated with 0.1% TFA containing 4 M urea, the modified enzyme was maintained on ice for about 1 h. The solution was centrifuged before being injected onto a reverse-phase Vydac C_4 column (0.46 × 25 cm) using a Varian model 5000 HPLC system equilibrated with 0.1% TFA in H_2O (solvent A). With solvent B as 0.075% TFA in acetonitrile, a linear gradient was started from 0% to 30% solvent B over 20 min, after the column was washed with solvent A for 10 min. Then another gradient continued to 55% solvent B in the next 100 min, followed by a final gradient to 100% solvent B in the next 30 min. The flow rate was 1 mL/min. Fractions of 1 mL were collected, the effluents were monitored at 220 nm, and 50-μL aliquots from the fractions were tested for radioactivity, as described above.

Subunits could also be separated by HPLC after prior treatment with 4% sodium dodecyl sulfate (SDS).

Separation of $\alpha_2\beta$ and γ Subunits by HPLC for Renaturation Experiments. NAD-dependent isocitrate dehydrogenase (1 mL, 0.8 mg/mL) was dialyzed overnight at 4 °C against 50 mM Mes buffer, pH 6.0, containing 20% glycerol, 0.4 mM $MnSO_4$, and 0.1 mM DTT. The dialyzed enzyme was centrifuged at 14 000 rpm before injection onto a reverse-phase Vydac C_4 column. The HPLC solutions and separation program are the same as those described above for urea-treated enzyme, except that the initial linear gradient from 0% to 30% solvent B was established over 30 min. For separation of $\alpha_2\beta$ and γ subunits from AMPS-BDB-modified isocitrate dehydrogenase, the enzyme (1 mL, 0.8 mg/mL) was dialyzed overnight at 4 °C against 50 mM PIPES, pH 7.0, containing 20% glycerol and 0.2 mM $MnSO_4$. The AMPS-BDB in water was added in two aliquots to yield a final concentration of 0.5 mM AMPS-BDB and 0.6 mg/mL enzyme. At various times, 5-μL aliquots were withdrawn to assay for the residual enzymatic activity. After the enzyme had reached a residual activity of 30−35%, the reaction mixture was dialyzed overnight at 4 °C against 50 mM Mes buffer, pH 6.0, containing 20% glycerol, 0.4 mM $MnSO_4$, and 0.1 mM DTT. The separation of modified $\alpha_2\beta$ and γ subunits on a C_4 column using HPLC was accomplished under the same conditions as those for unmodified NAD-isocitrate dehydrogenase under nondenaturing conditions. Two major peaks, as monitored at 220 nm, were collected and lyophilized separately. The relative amount of protein in each peak was determined from absorbance at 200 nm. The separated γ and $\alpha_2\beta$ subunit samples were stored at −75 °C before renaturation.

Renaturation of $\alpha_2\beta$ and γ Subunits from Unmodified and AMPS-BDB-Modified Enzyme. The separated γ and $\alpha_2\beta$ subunits from either unmodified or AMPS-BDB-modified enzyme were redissolved separately in 400 μL of 50 mM Mes buffer, pH 6.0, containing 20% glycerol, 2.4 mM $MnSO_4$, 0.1 mM DTT, and 10 mM isocitrate. Two sets of 50 μL of $\alpha_2\beta$ subunit were mixed with 100 μL and 150 μL of redissolved γ subunits (in a constant total volume of 200 μL) to yield $\alpha_2\beta$:γ ratios of 1:2 and 1:3. The mixtures were incubated at 25 °C for 1 h. A 10-μL aliquot of the freshly prepared detergent tetradecylammonium bromide (TTAB) solution (100 mM) was added to each subunit mixture to yield a final concentration of 6 mM TTAB. After 30 min at 25 °C, 20 μL of 200 mM cyclodextrin was introduced into the mixture to yield a final concentration of 16 mM methyl-β-cyclodextrin. This procedure is based on the method of Rozema and Gellman (*18*). The mixtures of $\alpha_2\beta$ and γ subunits, with both modified and unmodified subunits, were further incubated at 25 °C for another hour. The mixtures were then centrifuged at 14 000 rpm; 100-μL aliquots were withdrawn and assayed spectrophotometrically for isocitrate dehydrogenase activity.

Chemical Cleavage and Purification of Peptides from Separated Subunits of Modified Enzyme. Subunits α, β, and γ, after separation using reverse-phase C_4 HPLC, were lyophilized and redissolved in 0.5 mL of 70% formic acid. CNBr was added to the mixture at a 200:1 (mol/mol) ratio in accordance with the number of methionines in each subunit (*3*). After the enzyme was maintained in the dark for 24 hours at 4 °C, deionized water (5 mL) was added. The sample was then lyophilized and redissolved in 0.1% TFA in H_2O (1 mL) before being subjected to C_{18} reverse-phase HPLC. The different peptides resulting from CNBr chemical cleavage were separated by using the following solvent system: 0.1% TFA in water as solvent A and 0.075% TFA in acetonitrile as solvent B. For α and "β" subunits, after elution for 10 min with solvent A, a linear gradient was applied from 100% solvent A to 35% solvent B over 70 min, followed by another gradient to 40% solvent B over 50 min and to 100% solvent B in 20 min. For γ subunit, after elution for 10 min with solvent A, a linear gradient was applied from 100% solvent A to 30% solvent B over 60 min, followed by another gradient to 45% solvent B over 60 min and to 100% solvent B in 20 min. The separated radioactive peaks obtained by reverse-phase HPLC were further purified by HPLC with a TSK-2000 gel filtration column. The lyophilized peaks were dissolved in 0.5 mL of 0.1% TFA in H_2O (solvent A) and then applied to a TSK column which was equilibrated with solvent A. The column was eluted isocratically with solvent A, and fractions of 0.5 mL were collected. Aliquots (0.25 mL) of the fractions were tested for radioactivity.

Further Digestion with Pepsin and Separation of Peptides. Since the fragments obtained from the CNBr cleavage are long peptides (more than 60 amino acids), it was not easy to identify conclusively the modified amino acids. Therefore, pepsin digestion was carried out after the CNBr chemical cleavage. The radioactive fractions obtained from the CNBr peptides purified by HPLC were pooled and lyophilized, and digestion was conducted for 1 h at 25 °C in 0.1% TFA/H_2O, pH 2.0, with 5.0% (w/w) pepsin. Digests were separated by reverse-phase HPLC on a C_{18} column on the same day, at an elution rate of 1 mL/min. The column was eluted with solvent A for 10 min, followed by a linear gradient from solvent A to 10% solvent B in the next 20 min followed by a linear gradient to 25% solvent B between 30 min and 130 min and by a linear gradient to 35% solvent B in the next 20 min. Radioactivity was measured for each fraction.

Analysis of Radioactively Labeled Peptides. The purified peptides were sequenced by using an Applied Biosystems automated gas-phase protein/peptide sequencer, model 470A, equipped with an on-line PTH amino acid analyzer, model 120, and computer, model 900A. About 50−300 pmol of peptide was applied to the sequencer for analysis.

Reaction of Pig Heart NAD-Dependent Isocitrate Dehydrogenase with Phenylglyoxal in the Absence and Presence of Protecting Ligands. Enzyme (0.2 mg/mL) was preincubated in 0.1 M HEPES buffer, pH 7.4, containing 20% glycerol and 0.4 mM $MnSO_4$ at 30 °C for 20 min. To start the reaction, $NaHCO_3$ and phenylglyoxal in water were added at the same time to yield a final concentration of 40 mM $NaHCO_3$ and final concentrations of phenylglyoxal from 0.1 to 6 mM. In the protection studies, various concentrations of substrates, coenzymes, and regulatory compounds were preincubated with enzyme for 20 min prior to the addition of phenylglyoxal and $NaHCO_3$ (the final concentration of phenylglyoxal was 2 mM). The control enzyme was subjected to the same conditions but in the absence of phenylglyoxal and protecting ligands. Aliquots were withdrawn during the reaction at various times and assayed spectrophotometrically for isocitrate dehydrogenase activity.

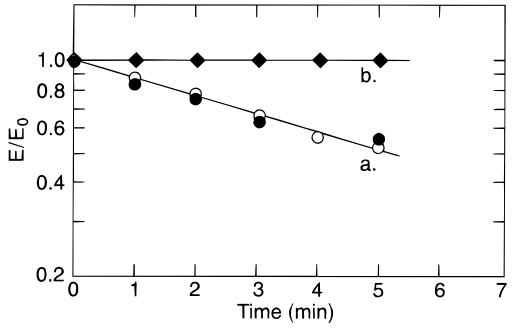

FIGURE 1: Reaction of NAD-dependent isocitrate dehydrogenase with AMPS-BDB in the absence or presence of isocitrate (20 mM) plus $MnSO_4$. Enzyme (0.1 mg/mL) was incubated with 0.4 mM AMPS-BDB at 25 °C in 50 mM PIPES, pH 7.0, containing 20% glycerol and 0.2 mM $MnSO_4$ either in the absence (a) or in the presence (b) of 20 mM isocitrate and additional 1 mM $MnSO_4$. The open and closed circles represent different experiments. At the indicated times, aliquots were withdrawn and assayed as described under Experimental Procedures. The rate constant was determined from the experimental data for the first 5 min to minimize the effect of the decomposition of AMPS-BDB.

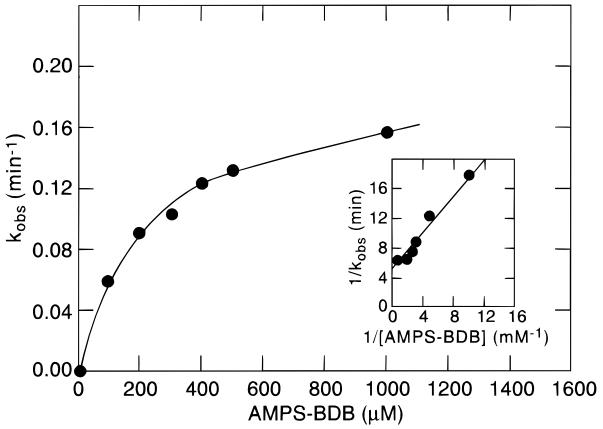

FIGURE 2: Dependence of the pseudo-first-order rate constants for reaction of isocitrate dehydrogenase as a function of the concentration of AMPS-BDB. The enzyme (0.1 mg/mL) was incubated with reagent concentrations from 0.1 to 1.0 mM at 25 °C in PIPES buffer, pH 7.0, and the rate constants were determined as described under Experimental Procedures. At each concentration, the rate constant was determined from the first 5 min of the reaction. The reciprocal plot in the inset allows the calculation of K_I and k_{max}, as described in the text.

RESULTS

Kinetics of Reaction of Isocitrate Dehydrogenase with AMPS-BDB. Incubation of pig heart isocitrate dehydrogenase with 0.4 mM AMPS-BDB in 50 mM PIPES buffer, pH 7.0, containing 0.2 mM Mn^{2+} and 20% glycerol results in a time-dependent inactivation (Figure 1, curve a), while there is no activity loss observed under the same incubation conditions in the absence of AMPS-BDB. Curvature is noted in the plots of ln E/E_0 vs time after 5 min of inactivation, and eventually the activity reaches a constant level dependent on the initial concentration of AMPS-BDB. However, the addition of a second aliquot of AMPS-BDB to the reaction mixture causes further inactivation, reducing the residual activity to 10% or less. AMPS-BDB is expected to undergo decomposition in aqueous solution with release of bromide ion since the bromodioxobutyl moiety is highly reactive; indeed, the half life for decomposition of AMPS-BDB has been measured as 16 min under similar conditions (*14*). The curvature observed in the plots of ln E/E_0 vs time after 5 min of inactivation can therefore be attributed to reagent decomposition, and the pseudo-first-order rate constant for the inactivation of isocitrate dehydrogenase by 0.4 mM AMPS-BDB was calculated from data obtained from the first 5 min of the reaction to minimize the effect of reagent decomposition.

Isocitrate dehydrogenase was incubated with a range of 0.1−1.0 mM AMPS-BDB, and k_{obs} was determined from data obtained in the first 5 min. A nonlinear dependence of the rate constant was observed over the range (Figure 2). This saturation kinetics indicates the reversible formation of an AMPS-BDB−enzyme complex prior to the irreversible modification, a result consistent with the action of AMPS-BDB as an affinity label for isocitrate dehydrogenase. The data can be analyzed according to the equation

$$1/k_{obs} = 1/k_{max} + (K_I/k_{max})(1/[R]) \quad (1)$$

where [R] represents the concentration of AMPS-BDB, k_{max} is the maximum rate constant at the saturating concentration of AMPS-BDB, and $K_I = (k_{-1} + k_{max})/k_1$, which is the apparent dissociation constant of the enzyme−reagent complex. From the reciprocal plot of $1/k_{obs}$ vs 1/[AMPS-BDB], we can calculate $k_{max} = 0.19$ min^{-1} and $K_I = 0.23$ mM.

Table 1: Effect of Ligands on the Rate of Inactivation by 0.4 mM AMPS-BDB[a]

line	additions to reaction mixture	k_{+L}/k_{-L}[b]
1	none	1.00
2	Mn^{2+} (1 mM)	0.88
3	ADP (1 mM)	0.53
4	ADP (1 mM) + Mn^{2+} (1 mM)	0.40
5	NAD (2 mM)	0.82
6	NAD (5 mM)	0.81
7	NAD (5 mM) + Mn^{2+} (1 mM)	0.47
8	NADH (5 mM)	0.47
9	NADH (5 mM) + Mn^{2+} (1 mM)	0.27
10	NADPH (5 mM)	0.16
11	NADPH (5 mM) + Mn^{2+} (1 mM)	0.16
12	isocitrate (1 mM) + Mn^{2+} (1 mM)	0.79
13	isocitrate (2 mM) + Mn^{2+} (1 mM)	0.42
14	isocitrate (5 mM) + Mn^{2+} (1 mM)	0.22
15	isocitrate (20 mM) + Mn^{2+} (1 mM)	0.02
16	isocitrate (20 mM) + EDTA (1 mM)	0.33
17	isocitrate (1 mM) + ADP (1 mM) + Mn^{2+} (1 mM)	0.07

[a] Isocitrate dehydrogenase (0.1 mg/mL) was preincubated for 20 min at 25 °C in 50 mM PIPES buffer, pH 7.0, containing 0.2 mM $MnSO_4$ and 20% glycerol. The AMPS-BDB was then added to yield a concentration of 0.4 mM. All the reaction mixtures contained 0.2 mM Mn2+ before the addition of ligands. [b] The rate constants are expressed as the ratio of the rate constant determined in the presence of ligands to that measured in the absence of ligands (k_{+L}/k_{-L}).

Effect of Ligands on the Rate of Inactivation of Isocitrate Dehydrogenase by 0.4 mM AMPS-BDB. Table 1 shows the effect of various ligands on the inactivation rate of the enzyme by 0.4 mM AMPS-BDB. The allosteric regulator ADP causes only a 2-fold decrease in the rate constant for inactivation despite the fact that the concentration of ADP is high relative to its known dissociation constant (*4*). The coenzymes NAD and NADH also have relatively small effects on the rate constant (Table 1, lines 5−9), although they are present in the reaction mixture at concentrations that saturate the enzyme (*4*, *5*). These results show that modification by the reagent does not occur at either the catalytic coenzyme site or at the allosteric ADP site.

The reduced coenzyme NADPH, an inhibitory nucleotide of the NAD-dependent isocitrate dehydrogenase, causes a somewhat greater decrease in the inactivation rate constant (Table 1, lines 10 and 11). However, the rate constant is never reduced to zero, despite the fact that the NADPH concentration is high relative to its dissociation constant (*5*). Thus, it is unlikely that the target of AMPS-BDB is directly within the NADPH site. We have previously shown that isocitrate displaces NADPH from isocitrate dehydrogenase, although this effect is probably caused indirectly by isocitrate binding at the active site (*5*).

The substrate isocitrate causes the most striking decrease in the inactivation by AMPS-BDB. As the concentration of substrate isocitrate is increased from 1 to 20 mM (Table 1, lines 12−15), the inactivation rate is progressively decreased. The enzyme is almost completely protected by 20 mM isocitrate and 1 mM Mn^{2+} against activation. It appears that AMPS-BDB reacts at the substrate isocitrate site.

The addition of 1 mM EDTA to chelate the free metal ions (Table 1, line 16) decreases the protection by isocitrate against inactivation by AMPS-BDB; this result is consistent with the known influence of metal ion in tightening the binding of isocitrate to enzyme (*4*). Although 1 mM isocitrate by itself has little protective effect (Table 1, line 12), in the presence of 1 mM ADP the same concentration of isocitrate yields almost complete protection against inactivation (Table 1, line 17). This result is consistent with the effect of ADP in enhancing the affinity of enzyme for Mn^{2+} and isocitrate (*4*).

Effect of ADP on the K_m for Isocitrate of Native and AMPS-BDB-Modified Isocitrate Dehydrogenase. The NAD-dependent isocitrate dehydrogenase is known to exhibits a K_m for isocitrate which is decreased by the allosteric activator, ADP (*6*). For example, the native enzyme in the present studies exhibited a K_m of 2.5 mM for isocitrate in the absence of ADP. An 8-fold decrease in the K_m for isocitrate to 0.33 mM was observed in the presence of 1 mM ADP. For AMPS-BDB-modified enzyme with 25% residual activity, the K_m for isocitrate was measured as 5 mM (twice that of the control enzyme), and this K_m was reduced to 1 mM in the presence of 1 mM ADP. The V_{max} was not changed for either enzyme by the addition of ADP. Therefore, although the modification by AMPS-BDB causes a small increase in the Michaelis constant for isocitrate, it does not affect the ability of ADP to strengthen the isocitrate−enzyme interaction.

Incorporation of AMPS-BDB into NAD-Dependent Isocitrate Dehydrogenase. The incorporation of reagent was determined using the radioactively labeled reagent [2-^{3}H]-AMPS-BDB. Upon incubation of enzyme with 0.4 mM [2-^{3}H]AMPS-BDB for 60 min, the modified enzyme exhibited 35% residual activity and contained 1.78 mol of reagent/mol of average subunit. In the presence of the protective ligands 20 mM isocitrate and 1 mM Mn^{2+}, the residual activity was 100%, and 1.3 mol of reagent/mol of average subunit was incorporated. Thus, the difference in incorporation between modified and protected enzyme was about 0.5 mol of reagent/mol of enzyme subunit, or 2 mol/enzyme tetramer. Extending the incubation time from 60 to 120 min did not change the measured incorporation, probably because of the rapid decomposition of AMPS-BDB.

Treatment of unmodified control enzyme and AMPS-BDB-modified enzyme with [^{3}H]$NaBH_4$ yielded no difference in measurable [^{3}H] between the two enzymes. These results suggest that the diketo groups of the enzyme-bound reagent are unavailable for reduction and that, therefore, the reaction of isocitrate dehydrogenase with AMPS-BDB may directly involve the two carbonyl groups of the AMPS-BDB.

Isolation of Modified Subunits after 4 M Urea Treatment. Enzyme modified with [2-^{3}H]AMPS-BDB in the absence or presence of 20 mM isocitrate and 1 mM Mn^{2+}, as described in Experimental Procedures, was incubated in 4 M urea for 1 h to dissociate the multimers before being injected onto a C_4 column. Figure 3 indicates the distribution of radioactivity in each subunit type for the AMPS-BDB-modified enzymes prepared in the absence (panel A) and presence (panel B) of the protective ligands. Three major radioactive areas are observed in Figure 3A, derived from inactive enzyme. These radioactive peaks are all decreased in enzyme modified in the presence of isocitrate and Mn^{2+} (Figure 3B). The α, *β*, and *γ* subunits differ in their N-terminal sequences (*3*), and the subunits in each radioactive peak were identified by N-terminal sequencing. The microheterogeneity of each subunit group, each of which has the same N-terminus, has also been observed by isoelectric focusing electrophoresis (*19*); it may be due to differences in the extent of amidation of the acidic amino acids. The peak in fractions 72−82 contains only *γ* subunit, while fractions 99−108 contain only α subunit.

The peak in fractions 90−98 (labeled as "*β*" subunit) contains *β* subunit plus non-dissociated enzyme. These fractions were pooled, lyophilized, and redissolved in 0.1% trilfluoroacetic acid containing 4 M urea. After incubation at 0 °C for 45 min, the solution was again subjected to chromatography in the solvent system shown in Figure 3. The absorbance at 220 nm was distributed over the α, *β*, and *γ* regions; however, the radioactivity was associated with only the α and *γ* peaks. These results indicate that most of the radioactivity initially observed in this region derived from the residual undissociated enzyme. Thus, radioactive AMPS-BDB reacts predominantly with the α and *γ* subunits.

Reversible Separation of $\alpha_2\beta$ and γ Subunits of NAD-Dependent Isocitrate Dehydrogenase. In contrast to the nearly complete dissociation of subunits of enzyme after treatment of the enzyme with 4 M urea (Figure 3), when the enzyme in buffer is rapidly injected onto an HPLC column equilibrated with 0.1% trifluoroacetic acid (Figure 4), only *γ* subunit is separated from the $\alpha_2\beta$ complex. Figure 4 illustrates the separation of subunits from AMPS-BDB-modified isocitrate dehydrogenase, indicated by the peptide absorbance at 220 nm. Two distinct UV peaks are observed between 43% and 50% acetonitrile, with *γ* subunit eluting first (fractions 82−87), followed by $\alpha_2\beta$ complex (fractions 102−114). The identification of the subunit types was verified by N-terminal sequencing, and the ratio between the amounts of protein in the two peaks is 1:3, as measured by $A_{220\ nm}$. The separation of *γ* subunit from the $\alpha_2\beta$ complex of unmodified enzyme was accomplished under the same conditions as for AMPS-BDB-modified enzyme.

Rozema and Gellman (*18*) have described the use detergents and cyclodextrin as "artificial chaperones" to facilitate the refolding of denatured protein. We have applied this

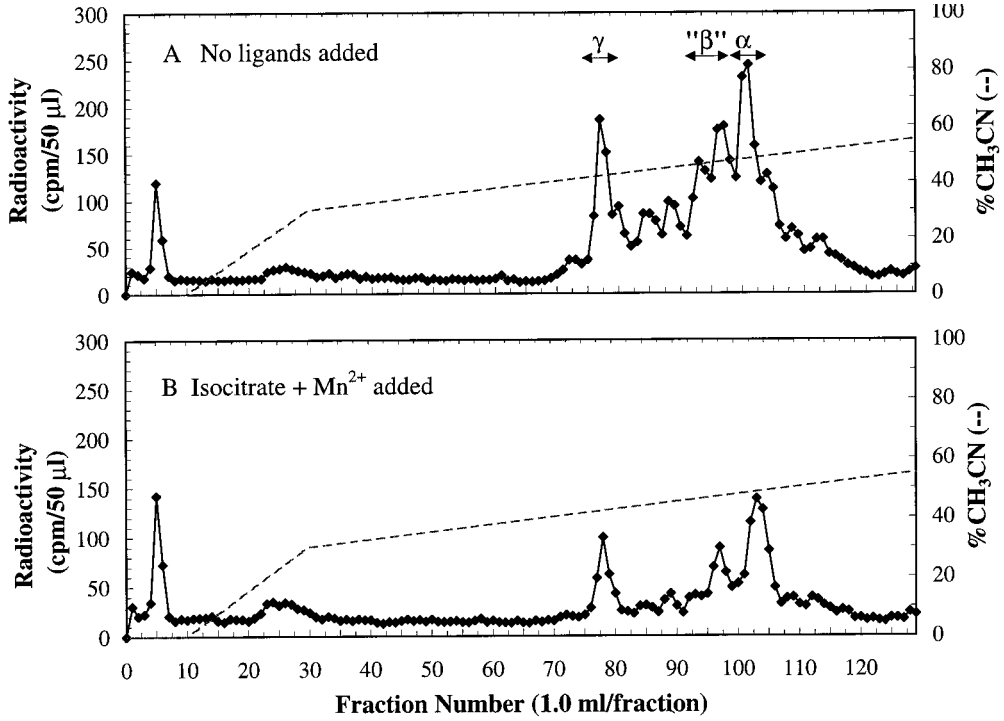

FIGURE 3: Fractionation of AMPS-BDB-modified α, *β*, and *γ* subunits of pig heart NAD-dependent isocitrate dehydrogenase by reverse-phase HPLC. After the treatment with 4 M urea, samples were applied to a C_4 column, equilibrated with 0.1% trifluoroacetic acid, and eluted using a gradient in acetonitrile, as described in Experimental Procedures. (A) Sample modified for 2 h in the absence of ligands; (B) sample modified for 2 h in the presence of 20 mM isocitrate and 1 mM $MnSO_4$.

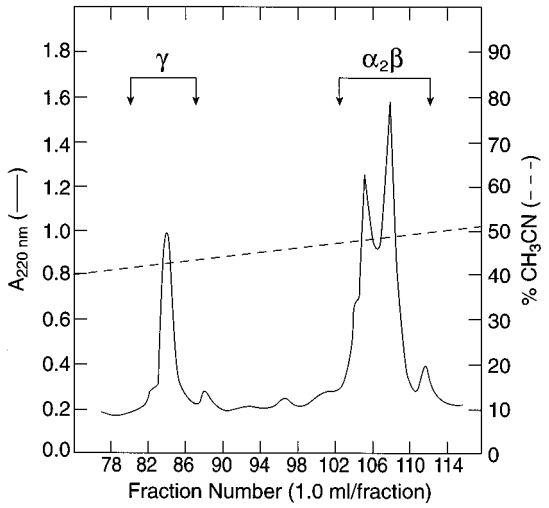

FIGURE 4: Separation of $\alpha_2\beta$ complex from *γ* subunit of AMPS-BDB-modified isocitrate dehydrogenase by HPLC (C_4 column) without prior denaturation of enzyme, as described in Experimental Procedures.

technique in refolding the denatured $\alpha_2\beta$ and *γ* subunits (both alone and in combination) which were separated from unmodified enzyme or from enzyme modified by AMPS-BDB. The renaturation was carried out at pH 6.0 in the presence of the substrate isocitrate and Mn^{2+} using tetradecylammonium bromide as the detergent followed by the addition of methyl-*β*-cyclodextrin. Table 2, column A, demonstrates that the isolated $\alpha_2\beta$ complex of unmodified enzyme exhibits some catalytic activity, while the separated *γ* subunit has no detectable activity. Addition of *γ* subunit (at ratios of 2:1 and 3:1) to the $\alpha_2\beta$ complex of unmodified enzyme enhances the observed catalytic activity as much as 7-fold.

To evaluate whether inactivation of isocitrate dehydrogenase by AMPS-BDB was caused by reaction with α or *γ* subunit, we incubated either unmodified $\alpha_2\beta$ complex with modified *γ* complex (Table 2, column B) or the same amount of modified $\alpha_2\beta$ complex with unmodified *γ* subunit (Table 2, column C). Comparison of columns A and B reveals that *γ* subunit, whether it is unmodified or modified by AMPS-BDB, produces about the same 7-fold activation of the unmodified $\alpha_2\beta$ complex; thus, modification of *γ* subunit does not impair its ability to stimulate catalytic activity. In contrast, AMPS-BDB-modified $\alpha_2\beta$ complex is not activated by either unmodified or modified *γ* subunit (Table 2, column C). These results indicate that, although AMPS-BDB reacts with both α and *γ* subunits, it is covalent modification of α subunit that is responsible for inactivation.

Isolation and Analysis of Peptides from Chemical Cleavage and Proteolysis of Radioactively Labeled α *Subunits.* CNBr cleavage of α subunit results in one major radioactive peak from the modified enzyme, as detected on HPLC using a C_{18} column, while in the protected samples, the peak is greatly reduced (data not shown). Table 3, column A, reports the amino acid sequences of representative peptides in this radioactive peak resulting from cleavage of modified α subunit by CNBr, following further purification by gel filtration on a TSK-2000 column. One major peptide (peptide I) is found, starting from Asn^{84}; the expected ending residue for this peptide is Met^{177}. Total radioactivity applied to the sequencer was 220 pmol based on the calculation from the radioactive pattern on the chromatogram. Since peptide I has about 90 amino acids, it is necessary to prepare smaller fragments to identify the modified residues.

Pepsin digestion of this long cyanogen bromide peptide yielded three major radioactive peaks. Figure 5 illustrates the radioactivity pattern from the pepsin digest of α subunit

Table 2: Effect on Activity of Recombination of Unmodified Subunits and Subunits Modified by AMPS-BDB[a]

(A) unmodified $\alpha_2\beta$ + unmodified γ		(B) unmodified $\alpha_2\beta$ + modified γ		(C) modified $\alpha_2\beta$ + either unmodified or modified γ	
subunit types	activity of subunit type[b] / activity of $(\alpha_2\beta)_u$	subunit types	activity of subunit type[b] / activity of $(\alpha_2\beta)_u$	subunit types	activity of subunit type[b] / activity of $(\alpha_2\beta)_u$
$(\alpha_2\beta)_u$	1	$(\alpha_2\beta)_u$	1	$(\alpha_2\beta)_u$	1
				$(\alpha_2\beta)_m$	0.2
$(\gamma)_u$	0	$(\gamma)_m$	0	$(\gamma)_u$	0
$(\alpha_2\beta)_u + 2(\gamma)_u$	5.8	$(\alpha_2\beta)_u + 2(\gamma)_m$	5.4	$(\alpha_2\beta)_m + 3(\gamma)_u$	0.6
$(\alpha_2\beta)_u + 3(\gamma)_u$	7.0	$(\alpha_2\beta)_u + 3(\gamma)_m$	7.3	$(\alpha_2\beta)_m + 3(\gamma)_m$	0.2

[a] The $\alpha_2\beta$ and γ subunits were separated as illustrated in Figure 4 and renatured under the conditions described in Experimental Procedures. The subscript "u" designates unmodified subunits, and "m" designates subunits isolated from AMPS-BDB-modified enzyme. [b] The activities of $(\alpha_2\beta)_u$ are expressed as nmol of NAD/min/mL of $\alpha_2\beta$ and ranged from 12 to 40. The activities of other subunit types or combinations of subunits are indicated relative to that of the $(\alpha_2\beta)_u$ used in the particular experiment.

Table 3: Amino Acid Sequences of Peptides in α Subunits Modified by AMPS-BDB

	(A) CNBr 139−140, peptide I	(B) pepsin−CNBr 139−140 (unprotected)[b]		(C) pepsin 139−140 (protected),[b]
cycle	(pmol)	fraction 90−92 (pmol)	fractions 97 + 98 (pmol)	fraction 97 (pmol)
1	Asn^{84} (257)	Leu (1369)	Tyr (526)	Tyr (166)
2	Leu (291)	**Arg^{88} (66)**	Ala (430)	Ala (103)
3	Leu (319)	Lys (883)	Asn (191)	Asn (81)
4	Leu (457)	Thr (455)	Val (233)	Val (51)
5	**Arg^{88} (67)**	Phe (901)	**Arg^{98} (50)**	**Arg^{98} (26)**
6	Lys (214)	Asp (353)	Pro (172)	Pro (51)
7	Thr (93)	Leu (266)	NEM-Cys	NEM-Cys
8	Phe (124)		Val (196)	Val (34)
9	Asp (100)		Ser (59)	Ser (7)
10	Leu (112)		Ile (121)	Ile (121)
11	Tyr (105)		Glu (66)	Glu (16)
12	Ala (101)		Gly (23)	Gly (8)
13	Asn (92)			
14	Val (98)			
15	**Arg^{98} (63)**			
16	Pro (67)			
17	NEM-Cys			
18	Val (81)			
19	Ser (25)			
20	Ile (58)			
21	Glu (33)			
22	Gly (31)			
23	Tyr (39)			
24	Lys (29)			
25	Thr^{108} (22)[a]			

[a] Sample was sequenced for only 25 cycles. [b] The peptides were separated by HPLC from a pepsin digest of modified α subunit, as indicated in Figure 5. These sequences are representative and were not derived from the same modified enzyme preparation. Thus, the amounts of the peptides do not represent the relative magnitude of the peaks shown in Figure 5.

of unprotected modified enzyme (panel A) and from the α subunit of samples prepared in the presence of the protectants isocitrate and Mn^{2+} (panel B). Table 3, column B, reports the amino acid sequences of peptides for unprotected sample. Fraction 90−92 (individually sequenced) yielded a 7 amino acid peptide: Leu^{87}−Leu^{93} (L**R**^{88}KTFDL). Analysis of fractions 97 + 98 yielded the amino acid sequence Tyr^{94}−Gly^{105} (YANV**R**^{98}PCVSIEG), as illustrated in Table 3, column B. In the pepsin digests for the protected samples, there were two radioactive peaks (Figure 5B) aligned with fractions 90 + 92 and fraction 97 in unprotected enzyme. However, the radioactivities of both peaks in protected enzyme are smaller than those in unprotected enzyme. The sequence of fraction 97 is a 12 amino acid peptide (Tyr^{94}−Gly^{105}) which contains Arg^{98} (Table 3, column C). This is the same peptide found in unprotected sample in fractions 97−98, but it is reduced in amount in the protected sample. Comparison of peptides from protected and unprotected samples suggests that AMPS-BDB reacts primarily with both Arg^{88} and Arg^{98} within the α subunit and that the modification of Arg^{88} and Arg^{98} is reduced by the presence of high isocitrate plus Mn^{2+}. We conclude that Arg^{88} and Arg^{98} are probably the important residues within the α subunit which are responsible for inactivation upon modification by AMPS-BDB; these residues are associated with the isocitrate binding site.

The modified γ subunit was also subjected to CNBr cleavage, followed by fractionation by HPLC. Only one radioactive peak was observed, which was purified further on a TSK2000 gel filtration column. Gas-phase sequencing showed that this peptide was Ala^{67}−Met^{186} of the γ subunit, a sizable peptide containing 10 arginine residues. Subsequent digestion of this CNBr peptide with pepsin yields suggestive evidence for the modification of Arg^{92} and Arg^{128}. Although we have not conclusively identified the labeled amino acids within this γ subunit CNBr peptide, this site is not important for enzyme function since the experiments involving the recombination of modified with unmodified subunits (Table 2) demonstrate that covalent modification of only the α subunit is associated with inactivation.

Inactivation of Pig Heart NAD-Dependent Isocitrate Dehydrogenase by Phenylglyoxal. Phenylglyoxal is a reagent known to react preferentially with arginine residues in proteins (*20*, *21*). Inactivation of NAD-dependent isocitrate dehydrogenase (0.2 mg/mL) with 2 mM phenylglyoxal at pH 7.4 causes a time-dependent inactivation, whereas, in the absence of phenylglyoxal, no change in activity is observed when the enzyme is incubated under identical conditions (data not shown).

Isocitrate dehydrogenase was incubated with 0.5−6.0 mM phenylglyoxal at pH 7, and the rate constants were determined from data obtained in the first hour. A linear dependence of the rate constant for inactivation on the reagent concentration was observed (Figure 6). This observation is indicative of a bimolecular chemical modification without the initial reversible formation of phenylglyoxal−enzyme complex prior to the irreversible modification, as is typical for affinity labeling.

Effect of Ligand on the Rate of Modification of Isocitrate Dehydrogenase by Phenylglyoxal. Table 4 shows the effects of various ligands on the rate constant for inactivation of

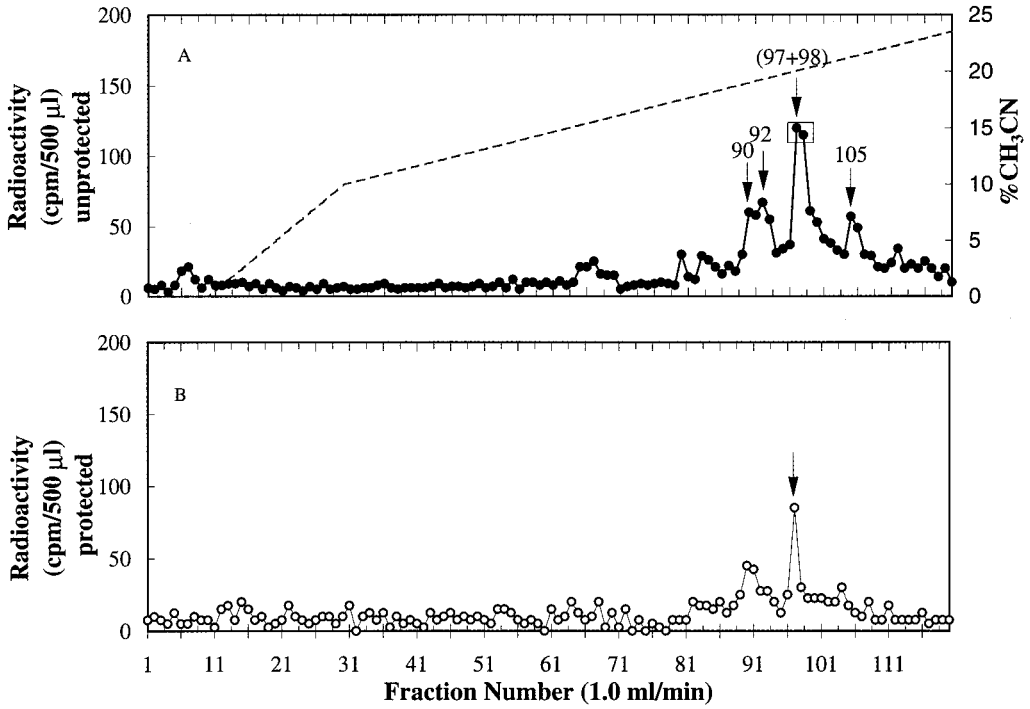

FIGURE 5: Separation of pepsin digests from α subunit CNBr cleavage fractions 139−140. The dashed line (- - -) indicates the elution gradient. The radioactivity pattern for the α subunit in the absence of ligands is represented by closed circles (●) in panel A, while open circles (○) represents that in the presence of 20 mM isocitrate and 1 mM $MnSO_4$ in panel B.

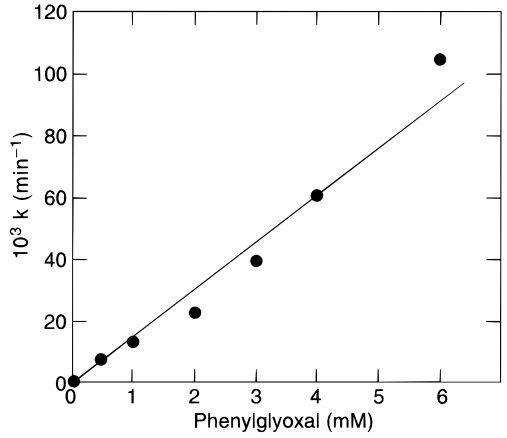

FIGURE 6: Rate constant for reaction of isocitrate dehydrogenase in HEPES buffer, pH 7.4, as a function of phenylglyoxal concentration. The rate constant was measured from the experimental data obtained in the first hour of the reaction.

the enzyme by 2 mM phenylglyoxal. In the presence of 2 mM Mn^{2+} alone little decrease in the inactivation rate is observed. The allosteric regulator ADP in the presence of 2 mM Mn^{2+} also causes less than a 2-fold decrease in rate constant for inactivation. Similarly, the coenzymes NAD and NADH provide minimal protection against inactivation. However, 2 mM Mn^{2+} and 1 mM isocitrate in the presence of 5 mM ADP gives more than 80% protection. This result is consistent with the lower K_m for isocitrate in the presence of ADP and metal. Mn^{2+} and 5 mM NADP or Mn^{2+} and 5 mM NADH results in some protection against inactivation. In contrast, striking protection against modification is provided by saturating concentrations of isocitrate (10 mM) in the presence of 2 mM Mn^{2+}. These results are similar to the effects of ligands on inactivation by AMPS-BDB (Table 1).

Table 4: Effect of Ligands on the Rate of Inactivation by Phenylglyoxal[a]

line	additions to reaction mixture	k_{+L}/k_{-L}[b]
1	none	1.00
2	Mn^{2+} (2 mM)	0.85
3	ADP (5 mM) + Mn^{2+} (2 mM)	0.69
4	NAD (5 mM) + Mn^{2+} (5 mM)	0.75
5	NADH (5 mM) + Mn^{2+} (2 mM)	0.60
6	NADPH (5 mM) + Mn^{2+} (2mM)	0.40
7	Isocitrate (10 mM) + Mn^{2+} (2 mM)	0
8	Isocitrate (10 mM)	0.38
9	Isocitrate (1 mM) + Mn^{2+} (2 mM)	0.87
10	Isocitrate (1 mM) + ADP (5 mM) + Mn^{2+} (2 mM)	0.19

[a] Isocitrate dehydrogenase (0.2 mg/mL) was incubated with 2.0 mM phenylglyoxal at 30 °C in 0.1 M HEPES buffer, pH 7.4, containing 20% glycerol, 0.4 mM $MnSO_4$, and 40 mM $NaHCO_3$. Additional ligands were included at the concentrations indicated. $MnSO_4$ was present at 0.4 mM in all the reaction mixtures before the addition of ligand. The rate constants were calculated as described under Experimental Procedures. [b] The rate constants are expressed as the ratio of the rate constant determined in the presence of ligands to that measured in the absence of ligands (k_{+L}/k_{-L}).

We have previously isolated the α subunit from isocitrate dehydrogenase labeled by [^{14}C]phenylglyoxal and have shown that Arg^{88} and Arg^{98} are the major amino acids within the α subunit which are modified by phenylglyoxal (*9*). Since isocitrate and Mn^{2+} are the best protectants of isocitrate dehydrogenase against *both* AMPS-BDB and phenylglyoxal, it is likely that both reagents target the same functional sites. These results strengthen the association of Arg^{88} and Arg^{98} of the α subunit with the isocitrate site of this enzyme.

DISCUSSION

The adenine nucleotide analogue adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] reacts covalently with pig heart NAD-dependent isocitrate dehydrogenase to pro-

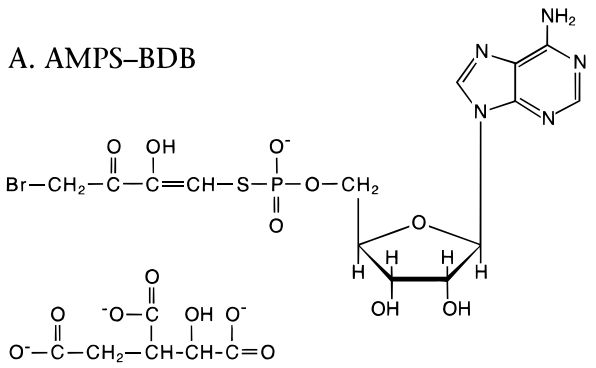

FIGURE 7: Schematic structures of (A) adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPS-BDB) and (B) isocitrate.

duce an irreversibly inactivated enzyme. It modifies predominantly α and γ subunits. The functional target site of AMPS-BDB is the isocitrate substrate site, as indicated by the protection studies. The rate constant for the inactivation exhibits a nonlinear dependence on the concentration of AMPS-BDB, indicating the formation of a reversible enzyme−reagent complex prior to irreversible modification. Even though AMPS-BDB is an ADP analogue, the regulatory site is not attacked by AMPS-BDB, since the modification of the enzyme does not affect the ability of ADP to decrease the Michaelis constant, K_m, for isocitrate. Moreover, 1 mM ADP alone and 1mM ADP with 1 mM Mn^{2+} provide only modest protection against inactivation. Instead, total protection against inactivation is provided by 20 mM isocitrate with 1 mM Mn^{2+}. Therefore, we conclude that AMPS-BDB does not react at the ADP regulatory site, but rather at the isocitrate substrate site.

This observation contrasts with the effects of other reactive nucleotide analogues on the NAD-dependent isocitrate dehydrogenase. The purine ring substituted compounds 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-diphosphate (*22*) and 2-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-diphosphate (*23*) cause enzyme inactivation; but in those cases total protection is provided by ADP, and it was concluded that both compounds act as affinity labels of the allosteric ADP site. Instead, AMPS-BDB reacts at the isocitrate site. The reaction target of AMPS-BDB may be understood in terms of a structural resemblance between isocitrate and the bromodioxobutyl moiety of the reagent. The dioxobutyl functional moiety of AMPS-BDB can exist in the enol form in aqueous solution, as shown in Figure 7. This form of the compound exhibits some similarity to isocitrate, with its hydroxyl and negatively charged carboxylates, and thus may bind at the isocitrate site, where it reacts with residues responsible for substrate binding, causing inactivation. It was previously found that AMPS-BDB reacts at the phosphenolpyruvate site of pyruvate kinase (*14*), and it was proposed that the enol form of the reagent functions as a bisubstrate analogue, mimicking both the nucleotide ADP and phosphoenolpyruvate positioned for phosphoryl transfer. In the case of isocitrate dehydrogenase, the effectiveness of AMPS-BDB may derive from its ability to occupy the proximal ADP site, although the covalent reaction occurs within the isocitrate site.

Bromodioxobutyl groups are potentially reactive with several nucleophiles in proteins, such as the side chains of cysteine, histidine, tyrosine, lysine, glutamic acid, and aspartic acid, while the dioxo group has the possibility of reacting with arginine residues (*24*). Recent studies using bromodioxobutyl reagents have provided several examples of arginine residues in proteins that have been attacked by the reagents (*15*, *25*, *26*). The reaction probably involves the guanidino side chain of arginine and the dioxo group to form a dihydroxy product. It has been reported that the product of AMPS and bovine liver glutamate dehydrogenase is not stable under neutral pH conditions (*15*). This observation is similar to the model study for the modification of arginine residues by cyclohexanedione (*27*). The formation of N^7,N^8-(1,2-dihydroxoycyclohex-1,2-ylene)-L-arginine occurs over the pH range 7−9; however, the product is most stable under acidic conditions and in borate buffers. In neutral or weakly alkaline solutions, free arginines are regenerated. The reaction products of AMPS-BDB with NAD-dependent isocitrate dehydrogenase exhibit the chemical properties of arginine derivatives. In its native state, the modified enzyme retains its radioactivity at neutral pH. However, denaturation of modified enzyme at pH 7 causes a large loss of radioactive AMPS-BDB and results in the regeneration of free arginine; this may be the reason that the amount of reagent incorporated into NAD-dependent isocitrate dehydrogenase is much lower when measured at pH 7.0 in PIPES buffer than it is at pH 2.0 in 0.1% TFA buffer (data not shown). The loss of radioactivity from the modified enzyme after denaturation at neutral pH makes proteolytic digestion by trypsin and thermolysin at pH 7.8 unsatisfactory. The effectiveness of CNBr chemical cleavage in 70% formic acid and of pepsin digestion at pH 2.0 in 0.1% TFA reflects the characteristics of the arginine and dioxo reagent adduct.

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E. coli            N-V-A-L-R119-Q-E-L-D-L-Y-I-C-L-R129-P-V-R-Y-Y-Q-G

Pig α Subunit      N-L-L-L-R88 -K-T-F-D-L-Y-A-N-V-R98 -P-C-V-S-I-E-G

Pig β Subunit      D-M-R-L-R99 -R-K-L-D-L-F-A-N-V-V109-H-V-K-S-L-P-G

Pig γ Subunit      N-N-I-L-R97 -T-S-L-D-L-Y-A-N-V-I107-H-C-K-S-L-P-G

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FIGURE 8: Comparison of the amino acid sequence of the *E. coli* NADP-dependent isocitrate dehydrogenase with those of the α, β, and γ subunits of pig heart NAD-dependent isocitrate dehydrogenase.

The lack of incorporation of tritium by the addition of [^{3}H]$NaBH_4$ to the modified enzyme gives additional evidence that the reaction of AMPS-BDB with NAD-ICDH occurs at arginine residues. The dioxo groups would be available for [^{3}H]$NaBH_4$ reduction if the reaction of enzyme with AMPS-BDB occurred by the displacement of the bromide. This [^{3}H]$NaBH_4$ reduction procedure has been successfully applied to several enzymes in which modification occurred at histidine or cysteine residues (*28*, *29*). In contrast, if dioxo groups are involved in the reaction, there would be no tritium incorporation upon treatment with [^{3}H]$NaBH_4$, as we found for the reaction between AMPS-BDB and NAD-dependent isocitrate dehydrogenase: no difference in tritium incorporation was observed between AMPS-BDB-modified enzyme, protected enzyme, and control enzyme not treated with AMPS-BDB.

Early studies of the chemical modification of pig heart NAD-dependent isocitrate dehydrogenase by butanedione implicated arginine residues in both the catalytic function and the allosteric activation by ADP (*30*). Isocitrate plus $MnSO_4$ yielded the best protection against inactivation. Although modified arginines were quantified in that study, it was not possible to isolate a labeled peptide.

There are numerous arginine residues located in NAD-dependent isocitrate dehydrogenase. However, in the α subunit the peptides containing Arg^{88} and Arg^{98} are labeled to the greatest extent. In the γ subunit, the modified arginines have been firmly localized only within the CNBr peptide 67−186. But the identification of the reaction site within the γ subunit is not important; the experiments on recombination of unmodified and AMPS-BDB-modified subunits clearly demonstrate that unmodified $\alpha_2\beta$ is activated equally well by modified and unmodified γ subunit. Thus, reaction of AMPS-BDB with the γ subunit has little or no effect on function. In contrast, the AMPS-BDB-reacted $\alpha_2\beta$ complex is not activated by either unmodified or modified γ subunit, demonstrating that covalent modification of the α subunit by AMPS-BDB causes the major loss of activity of the enzyme.

Direct binding studies show that 2 mol of isocitrate per tetramer bind tightly to the pig heart NAD-dependent isocitrate dehydrogenase (*4*). It is not known whether there is a specific subunit responsible for this tight binding. Our study indicates that the isocitrate binding sites are localized on the α subunit, of which there are two per $\alpha_2\beta\gamma$ tetramer. The difference in incorporation of [2-^{3}H]AMPS-BDB between the unprotected and protected enzymes is about 2 mol of reagent/mol of enzyme tetramer, suggesting that modification of two amino acid residues per tetramer is responsible for the inactivation.

The only crystal structure of isocitrate dehydrogenase which has been determined is the *Escherichia coli* NADP-dependent isocitrate dehydrogenase. Structures of the enzyme complexed with the coenzyme, NADP, and with magnesium isocitrate have been solved (*31*). The Mg^{2+}-isocitrate was found to bind in the pocket between the large and small domains of the enzyme. Figure 8 presents part of the sequence alignment of the α, β, and γ subunits of pig heart NAD-ICDH with *E. coli* NADP-dependent isocitrate dehydrogenase using the CLUSTAL program of PC/Gene (Intelligenetics). Arg^{88} and Arg^{98} of the α subunit are aligned, respectively, with Arg^{119} and Arg^{129} of the *E. coli* NADP-dependent isocitrate dehydrogenase. Both Arg^{119} and Arg^{129} of the *E. coli* enzyme have been shown to constitute part of the substrate binding site, where they interact with the α- and β-carboxylates of isocitrate (*31*). It seems likely that in the pig heart NAD-dependent isocitrate dehydrogenase Arg^{88} and Arg^{98} fulfill the analogous roles of facilitating the binding of isocitrate by interacting with the carboxylate groups. It may be that AMPS-BDB can react with either of these arginines on an α subunit, but once reaction has occurred on a given residue, reaction with another amino acid on that subunit is excluded. Thus reaction of Arg^{88} and Arg^{98} within a given subunit may be mutually exclusive.

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