Malate Dehydrogenase, Inhibition of Pig Heart Supernatant Enzyme by Iodoacetamide*

Thomas E. Aspray, George M. Riihimaki, and Raymond G. Wolfe

From the Chemistry Department, University of Oregon, Eugene, Oregon 97403

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Pig heart supernatant malate dehydrogenase is alkylated by 250 mM iodoacetamide at 37°C in pH 7.5 Tris/acetate buffer which is 0.05 M with respect to acetate to form enzyme with 1,3-dicarboxamidomethyl histidine, 3-carboxamidomethyl histidine, 1-carboxamidomethyl histidine, carboxamidomethyl cysteine, and carboxamidomethyl methionine. 1,3-Dicarboxamidomethyl histidine forms with a stoichiometry of 1/ enzyme subunit at a rate equaling the rate (approximately 1.24 ± 0.09 × 10^{-4} min^{-1}) of enzyme inactivation by iodoacetamide. All other amino acid derivatives form at a slower rate initially or continuously. The enzyme is protected against inactivation by iodoacetamide through NADH binding, suggesting that there is an "essential" histidine residue at or near the active site.

Mitochondrial and supernatant malate dehydrogenases, two structurally distinct enzymes, appear to be ubiquitous in eukaryotic cells. Although the two enzyme forms have been recognized for over 15 years, the relationship of their structural differences to their biological roles is not understood. This paper describes an attempt to understand this relationship through a study of the supernatant enzyme properties which can be compared with a previous study of the mitochondrial enzyme (1).

Each of the enzymes has two structurally very similar, if not identical, subunits (2-4). The differences in amino acid composition have been established (5-9). Inactivation of the mitochondrial enzyme is accompanied by the alkylation of cysteine (10-12), histidine (1, 11, 13, and 14), and arginine (14). Inactivation of the supernatant enzyme is accompanied by the alkylation of methionine (11 and 15) and histidine (16).

The mitochondrial and supernatant enzymes differ in sensitivity to inhibition and in the amino acids which are derivatized by various alkylating agents. For example, the mitochondrial enzyme reacts readily with 4,4'-bisdimethylamino-diphenylcarbinol (12), a reagent apparently highly specific for sulfhydryl groups, but the supernatant enzyme apparently fails to react with this reagent (17). Iodoacetate, but not iodoacetamide, is reported to react with the supernatant enzyme, but the mitochondrial enzyme reacts with both reagents (1, 18).

Although the two enzymes differ significantly in their reactivity with various alkylating reagents, it seems reasonable to expect some homology of structure in the vicinity of the active site. The observation that iodoacetamide reacts stoichiometrically with an active site histidine residue in the mitochondrial enzyme (1) led us to reinvestigate the reaction of this reagent with the supernatant enzyme under experimental conditions differing from those reported previously. The results are reported here.

**MATERIALS AND METHODS**

Pig heart supernatant malate dehydrogenase was isolated from pig hearts by the method of Gerding and Wolfe (8) modified to increase the quantity of enzyme obtained. This method routinely produces enzyme homogeneous by several criteria, and the preparations used in this work were found to be homogeneous by gel or cellulose/acetate strip electrophoresis at pH 6.9, 8.4, and 9.0. The enzyme activity was measured by the addition of 10 µl of appropriately diluted enzyme to 1.0 ml of assay solution containing 1.1 mM NAD and 0.1 M l-malate in 0.1 M glycine buffer of pH 10.0. The course of the reaction was recorded with the use of a Gilford model 220 spectrophotometer.

All inhibition studies were carried out in the presence of 250 mM iodoacetamide at 37°C at appropriate pH conditions. A pH dependence study was carried out from pH 5.6 to 9.1 in phosphate buffer of 0.2 ionic strength. The exact pH values are apparent in the data. Also, the rate of inactivation and the rate of alkylation of various amino acids were measured at pH 7.5 in Tris/acetate buffer which was 0.05 M with respect to acetate.

**Rationale**—Because several amino acids were alkylated by iodoacetamide under our experimental conditions, it was necessary to compare the rate of alkylation of each amino acid with the rate of inactivation in order to make the structure-function correlation. Consequently, the alkylation of each reacting amino acid was measured as a function of time with the use of [1,14C]iodoacetamide. The amino acids and amino acid derivatives were separated by ion exchange on an amino acid analyzer and the radioactivity of each alkylated amino acid was determined in parallel with the loss of enzyme activity at appropriate time intervals.

**Enzyme Alkylation with [1,14C]Iodoacetamide**—To 1.0 µg of enzyme in 0.5 ml of Tris/acetate buffer, 0.05 M with respect to acetate, was added an equal volume of 500 mM [1,14C]iodoacetamide (American/Searle). In order to obtain the required reagent concentration with a minimum amount of costly radioactive reagent, it was necessary to decrease the specific activity of the reagent from 57 mCi/mol to 230 µCi/mmol by the addition of nonradioactive iodoacetamide. The reaction mixture was kept at 37°C, and 200-µl samples were removed 19, 40, 88, 78, and 97 min after the start. The reaction was stopped in each sample by placing them in an ice bath and adding an equal volume of 0.2 M 2-mercaptoethanol. The catalytic activity was also measured in each of the series of samples before exhaustive dialysis against distilled water.

**Amino Acid Analysis and Radioactive Assay Technique**—The contents of each dialysis bag was quantitatively delivered into a reusable hydrolysis tube (Pierce). Before lyophilizing, 50 mM of noreleucine was added to each tube as an internal standard to correct for manipulative losses. After the tubes were evacuated and flushed with nitrogen, the protein samples were hydrolyzed for 4 hr in 6 N HCl at 110°C. Following hydrolysis, the HCl and water were removed under vacuum in the presence of solid NaOH in a vacuum desiccator. This drying step was repeated twice in order to remove all traces of volatile material. Each sample was then dissolved in 50 µl of 0.12 M sodium citrate buffer (pH 2.2) and 2,000 cpm of [14C]leucine was
added as a chromatography marker. The total hydrolysate was then applied to the long column of a Technicon TSM amino acid analyzer adjusted to a full scale sensitivity of 50 nmol. After passing through the 570 nm colorimeter cuvette the eluate stream was split 73% to a fraction collector and 27% was lost to the debubbler. Fractions of 0.75 ml were collected at a low rate of 1.3 ml/min directly into small disposable plastic vials (Evergreen). Because of the high salt content and large water volume in the samples to be counted, the gel-flow counting solution of Olson et al. (19) was used. This contained 1 liter of scintillation grade dioxane, 4.0 g of 2,5-diphenyloxazole (POPOP), 0.05 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP) (both from Sigma), 120 g of naphthalene (Baker), and an equal volume of dry Cab-O-Sil (from Portland Reagents Co., grade M-5). Cab-O-Sil is a gelling agent used to disperse and suspend the sample. The sample was then mixed by gentle swirling at room temperature (to prevent precipitation) and stored 20 h in the dark to allow phosphorescence to decay before counting. Counting was accomplished on a Nuclear Chicago model Isocap/300 liquid scintillation counter. Each sample was counted a minimum of 20 min in "program 2" (26C standard channels ratio) for low energy B counting. All counts were corrected for background radioactivity.

Identifying Amino Acid Derivatives—The background-corrected radioactive count for each sample was then plotted versus the sample number, as a nomogram. The elution nomogram was then aligned with the ninhydrin reaction elution profile from the recorder by superimposing the ninhydrin leucine peak on the leucine C-14 peak. The more relevant radioactive derivatives produced by alkylation radioactive count for each sample was then plotted versus the sample number, as a nomogram. The elution nomogram was then aligned with the ninhydrin reaction elution profile from the recorder by superimposing the ninhydrin leucine peak on the leucine C-14 peak. The more relevant radioactive derivatives produced by alkylation with [14C]iodoacetamide were identified by comparison with elution curves produced with known samples (1,3-dioxygenyl methyl) histidine, 1-carboxyethyl histidine, and 3-carboxymethyl histidine (Calbiochem). The amide derivatives are hydrolyzed to free carboxyl groups during hydrolysis of the protein in preparation for the amino acid analysis. For this reason, alkylated derivatives will be referred to as carboxymethyl or carboxamidomethyl derivatives as appropriate.

Two-dimensional paper chromatography was also used to verify the identification of the histidine and other derivatives produced by alkylation of the protein. The solvents used in paper chromatography were butanol/acetic acid/water (10:3:7), a modified solvent of Smith (20), and butanol/pyridine/water (1:1:1), a solvent of Smith et al. (21). The chromatogram was developed a second time, after drying, in the former solvent system. Amino acid spots were visualized with ninhydrin and some samples were stabilized with the copper nitrate spray (22). The RF values of the important derivatives of amino acids produced by alkylation of the protein and the identity of most of the unusual spots on the chromatogram were determined with the use of known amino acid derivatives.

Quantitating Each Sample—Because it was uncertain that a quantitative recovery of protein had been achieved in each sample, the protein present was calculated from the known amino acid composition (18) and the amounts of several stable amino acids (glycine, valine, alanine, and isoleucine), measured in each sample on the amino acid analyzer. Corrections were made for manipulative losses during hydrolysis with the use of norleucine as an internal standard. The radioactivity of each sample was then corrected to the same amount of protein for each of the eluted radioactive peaks. The radioactivity in each of the radioactive peaks was plotted as the log of activity versus time of alkylation to test for pseudo-first order rates of alkylation and for the rate of enzyme inactivation in the same experiment.

Treatment of Data—An accurate comparison of the rate of inactivation of the enzyme to the rate of alkylation of each derivatized amino acid is essential in order to make the structure-function correlation. Since the remaining activity of the enzyme during inactivation is to be compared with the amount of unalkylated "essential" amino acid residue, it is important to have an accurate estimate of the total amount of essential residue in each sample. Assuming the presence of one essential amino acid residue per subunit, the maximum possible radioactivity (Cmax) can be calculated from the known specific activity and the total protein, determined as described in the previous paragraph. The log of Cmax - C (C is radioactive at time "t") was plotted versus time to test for pseudo-first order behavior and to evaluate the alkylation rate constants.

RESULTS AND DISCUSSION

Fig. 1 shows the inactivation behavior of supernatant malate dehydrogenase by 250 mM iodoacetamide at 37°C and at pH 7.5 in Tris/acetate, 0.05 M with respect to acetate. It is known that the reaction is pseudo-first order in character through at least two log cycles. Fig. 2 shows the pH dependence of the inactivation rate in the same buffer at 37°C. It is apparent that the inactivation rate increases at alkaline pH and the rate profile is consistent with the involvement of two acid functional groups, one at an approximate pK of 7 and a second at about 8.8. The lower pK is in the range frequently ascribed to the histidine imidazolium group. Although the inactivation data seem to follow pseudo-first order kinetic behavior at pH 7.0 or less, we observe that the rate of inactivation increases with time at alkaline pH. The pH inactivation profile in Fig. 2 closely resembles that reported for the mitochondrial form of the enzyme by Anderton and Rabin (1).

Fig. 3 shows the protection against iodoacetamide inactivation by NADH, an indication that the inhibitor is reacting at or near the active site of the enzyme. This property also
parallels qualitatively that reported for the mitochondrial enzyme \(^{(1)}\).

Fig. 4 represents the amino acid analyzer elution nomogram (shaded) for the \(^{14}C\)-labeled enzyme which had been 95% inactivated by alkylation in 250 mM iodoacetamide. The superimposed ninhydrin-reacting elution peaks represent the amino acids eluted in the same column run. Radioactive nomogram peaks are identified by Roman numerals. Control experiments with a methionine peptide indicate that Peak I is an alkylated methionine degradation product. Peak II is 1,3-dicarboxymethyl histidine, Peak IV is 1-carboxymethyl histidine, and Peak V is 3-carboxymethyl histidine. These elution positions are consistent with similar data published by Lesco vac \(^{(18)}\). Peak III is the carboxymethyl sulfonium salt of methionine mixed with carboxymethyl cysteine. This peak had an alkylation rate much less than the rate of inactivation of the enzyme so its composition was not investigated in detail.

Weakly adsorbed substances show variation in the elution volume from the Auto Analyzer. This variation was found to relate to the manner of sample application and other unidentified variables. This variation accounts for the poor match between the radioactive and ninhydrin peaks in Fig. 4 corresponding to 1,3-dicarboxymethyl histidine (Peak II). Possible doubt about the identification of Peak II was eliminated through paper chromatography to be described below. The data are slightly complicated by the fact that 1,3-dicarboxymethyl and 1-carboxymethyl histidine undergo spontaneous decomposition which produces two closely eluting peaks in ion exchange chromatography (see Peak II) and double spots in chromatography (see below).

Peaks I, III, IV, and V all appear initially more slowly than Peak II (1,3-dicarboxymethyl histidine). Peaks I and IV appear in accordance with approximate first order behavior. Peaks III and V, on the other hand, appear biphasically with the alkylation rate increasing with time.

Fig. 5 depicts the separation of the same 95% inactivated enzyme hydrolysate that was analyzed by ion exchange chromatography (Fig. 4). It is apparent that five radioactive amino acid derivatives, which were identified by chromatography of known amino acid derivatives, are present. Three of the radioactive spots were identified as the three histidine derivatives mentioned before, the fourth spot has the same \(R_f\) value as carboxymethyl cysteine, and the fifth radioactive derivative is believed to be a methionine derivative. It was observed that 1,3-dicarboxymethyl histidine and 1-carboxymethyl histidine, as well as the commercial preparations of these derivatives, produce two partially resolved spots in the butanol/pyridine/water solvent system. The ion exchange and paper chromatography techniques are self-consistent in the identification of the histidine derivatives.

Fig. 6 indicates the rate of alkylation to form 1,3-dicarboxymethyl histidine and the rate of inactivation of the enzyme. The measured rates are \(1.23 \pm 0.09 \times 10^{-2}\) min\(^{-1}\) for alkylation and \(1.25 \pm 0.08 \times 10^{-2}\) min\(^{-1}\) for the inactivation. We conclude that the histidine residue which reacts with iodoacetamide with a stoichiometry of 1/subunit to form 1,3-dicarboxymethyl histidine is likely a functional group in catalysis. The stoichiometry of 1/subunit was established from the known specific activity of the \(^{14}C\)-iodoacetamide, the amount of protein, and the measured radioactivity of Peak II. The stoichiometry is also apparent in the data plotted in Fig. 7. It was found, after extrapolation from 95 to 100% inactivation, that Peak V (3-carboxymethyl histidine) contained half of the radioactivity measured in Peak II (1,3-dicarboxymethyl histidine). Allowing for the double alkylation of Peak II, this means that both
Malate Dehydrogenase Inhibition

FIG. 6. A comparison of the pseudo-first order inactivation (△) rate with the alkylation (○) rate forming 1,3-dicarboxymethyl histidine by 250 mM iodoacetamide at pH 7.5 in Tris/acetate buffer which was 0.05 M with respect to acetate. The lines are least squares best fit to the data of each type. The zero time activity value has been adjusted to correspond to the value of Cmax calculated from the measured amount of protein in the sample and the known specific activity of the [14C]iodoacetamide.

FIG. 7. A plot of per cent of the remaining activity versus the number of residues of 1,3-dicarboxymethyl histidine formed per subunit of supernatant enzyme, indicating that this derivative is formed with a stoichiometry of 1/subunit under experimental conditions given in the legend of Fig. 6.

derivatives form with a stoichiometry of 1, although the 3-carboxymethyl histidine achieves this only by accelerated alkylation late in the inactivation.

Because alkylated methionine undergoes relatively rapid decomposition, it is not possible to conclude from our data that this residue is not also involved in catalysis. It would appear that, although S-carboxymethyl (or carboxamidomethyl) methionine is formed with iodoacetate (15) or iodoacetamide (this work), the mechanisms of inhibition by iodoacetate and iodoacetamide may differ. This follows from our correlation of the rate of inhibition by iodoacetamide with the rate of alkylation of an “essential” histidine and the observation by Leskovac and Pfleiderer (15) that the rate of inhibition by iodoacetate is independent of pH in the range between 6 and 9. As our data show (Fig. 2), the inactivation rate with iodoacetamide is very pH-dependent.

Supernatant and mitochondrial malate dehydrogenases differ significantly in their reaction with iodoacetamide. The former enzyme is orders of magnitude more reactive with iodoacetamide, forming 3-carboxamidomethyl histidine in a pseudo-first order manner without forming measurable quantities of other amino acid derivatives. Mitochondrial enzyme is protected from alkylation through NADH binding in a manner consistent with known NADH binding properties. By comparison, the supernatant enzyme is protected from alkylation less by NADH than one would predict assuming that the critical alkylation of the enzyme-coenzyme complex is impossible. This perplexing observation requires further study.

We are aware of no other example in which double alkylation of single histidine residue has been reported in a protein. It is interesting that the double alkylation of one histidine residue occurs at a rate exceeding that of single derivatization of other histidine residues, and it implies a rather special environment.

REFERENCES

Malate dehydrogenase, inhibition of pig heart supernatant enzyme by iodoacetamide.

T E Aspray, G M Riihimaki and R G Wolfe


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