Identification of the Essential Cysteine Residue in the Active Site of Bovine Pyruvate Dehydrogenase*

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Pyruvate dehydrogenase (E1), the first catalytic component of the bovine pyruvate dehydrogenase complex, is composed of two nonidentical subunits in a tetrameric αβ2 form. The sulfhydryl-specific reagent N-ethylmaleimide (NEM) was used to identify the reactivities and function of cysteiny1 residues and subsequent identification of these residues in the active site of bovine E1. Treatment of E1 with 0.2 mM NEM resulted in loss (90%) of enzymatic activity; the inactivation followed bimolecular reaction kinetics. The inactivation was almost entirely prevented by thiamin pyrophosphate (TPP) and pyruvate; protection is probably due to formation of the hydroxyethylidene-TPP intermediate. To identify the reactive cysteiny1 residues in the active site region, the nonessential SH groups in E1 were first modified with NEM in the presence of TPP and pyruvate. After quenching with dithiothreitol and removal of the substrate and cofactor by dialysis, the modified E1 was treated with [14C]NEM to label the exposed cysteiny1 residue(s) in or near the active site region. The data indicate that NEM reacted in the active site region of the E1 component with a stoichiometry of 2 mol of [14C]NEM bound per mol of E1 tetramer. The initial rapid labeling of E1 with [14C]NEM established that incorporation was predominantly into the α subunit. A single radiolabeled peptide was isolated following V8 protease digestion of radiolabeled E1 by [14C]NEM. Sequence analysis of the labeled peptide derived from bovine E1 demonstrated that the labeled cysteiny1 residue was equivalent to Cys-62 in the α subunit (mature form) of human E1.

The pyruvate dehydrogenase (E1) component of the mammalian pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate in a two-step process. In the first step, it forms CO2 and hydroxyethylidene-thiamin pyrophosphate (HE=TPP) intermediate. The HE=TPP intermediate is then oxidized to an acetyl group with concomitant reduction and acetylation of the lipoyl group covalently linked to the dihydrolipoamide acetyltransferase component. This second step appears to be the rate-limiting step in the overall PDC reaction (Cate et al., 1980). The acetyl group and reducing equivalents formed are transferred sequentially from the dihydrolipoamide acetyltransferase to CoA and to the dihydrolipoamide dehydrogenase component, respectively (Reed, 1974). Mammalian E1 is composed of two nonidentical subunits α and β with a tetrameric structure (α2β2) and is regulated by reversible interconversion between an active nonphosphorylated form and an inactive phosphorylated form by specific phospho-E1α phosphatase and E1α kinase, respectively. The available evidence suggests that E1α plays a crucial role in catalysis (Roche and Reed, 1972; Stephens and Reed, 1985), and E1β is involved in the binding of E1 to the structural core of dihydrolipoamide acetyltransferase (Rahmatullah et al., 1989) and may participate in the second partial reaction of PDC (Roche and Reed, 1972).

From chemical modification studies, the amino acid residues which play important role in catalysis have been characterized in pigeon breast muscle E1 (Khailova et al., 1989) and Escherichia coli E1 (Schwartz and Reed, 1970). A more detailed investigation of the pigeon enzyme revealed the presence of a highly reactive cysteiny1 residue, located within or near the active site of E1 contributing directly to the transformation of reactants to products (Khailova et al., 1985). Flournoy and Frey (1989) suggested a nucleophilic role for cysteiny1 residues in E. coli E1. E1 requires reducing agents such as dithiothreitol or β-mercaptoethanol for maximal activity (Khailova et al., 1983) and is shown to be sensitive to thiol reagents (Schwartz and Reed, 1970). This evokes the question as to which cysteine residue(s) is essential for the catalytic activity and what are the optimal conditions for making this identification. Knowledge of the primary structure creates the potential for precise placement of critical cysteiny1 residues.

The primary structures of both α and β subunits of human E1 were deduced from their cDNA sequences (Ho et al., 1989; Ho and Patel, 1990). There are 13 and 6 cysteiny1 residues in the primary sequences of α and β subunits of human E1, respectively. We undertook a chemical modification study to identify the cysteine residue(s) that is likely to be involved in the catalysis of bovine E1 using TPP and pyruvate as protecting agents at the catalytic site. It was possible to specifically label a cysteine residue by radioactive NEM which caused inactivation of E1. The critical cysteiny1 residue was identified and was found to be equivalent to Cys-62 of human E1α. This report provides the first localization of cysteine residue at or near the active site of E1 component of the PDC.

EXPERIMENTAL PROCEDURES

Materials—NEM was purchased from Sigma. Acetonitrile was from Fisher. Trifluoroacetic acid and V8 protease were from Pierce Chemical Co. [1-14C]NEM (88.7 μCi/mmol) from Du Pont-New England Nuclear was diluted with unlabeled NEM and stored at −20 °C. Assay of E1 Activity—Bovine kidney PDC was purified as described previously (Roche and Cate, 1977). The PDC components were resolved

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according to established procedures (Linn et al., 1972; Roche and Cate, 1977). Reconstitution assays were conducted with an excess of E2 and E3 added together with E1 and the overall PDC activity was measured (Roche and Reed, 1972). Protein concentrations were determined spectrophotometrically using the Bio-Rad Protein assay reagent with bovine serum albumin as a standard.

RESULTS

Reaction of E1 with NEM—E1 was inactivated in a time-dependent manner when incubated with excess NEM. The loss in activity was pseudo first order at a given NEM level (Fig. 1). The linear relationship between $k_{app}$ min$^{-1}$ and [NEM] shows a simple bimolecular relationship for the reaction between enzyme and reagent (Fig. 1, inset). The slope of the curve yields a second order rate constant of 1.2 mmol$^{-1}$ min$^{-1}$.

Protection of E1 against Inactivation by NEM—Substrate and cofactor protection against NEM inactivation of E1 was evaluated and the results are shown in Fig. 2. Neither TPP nor pyruvate alone afforded protection against inactivation of E1 by NEM; however, TPP (50 mM) plus pyruvate (100 mM) allowed 70–80% of E1 activity to be retained even after a 20-min treatment with 0.2 mM NEM. It is likely that catalytic turnover led to the active site being occupied by the HE=TPP intermediate under these conditions.

Circular Dichroism—Circular dichroism spectra (190–260 nm) were collected for both the native and the modified E1 (results not shown). The data suggested that the secondary structure of E1 was not significantly perturbed by NEM modification.

Specific Radiolabeling of Essential Cysteine Residue—We have taken advantage of a two-step modification procedure (Tu and Weiner, 1988) to preferentially label a cysteine residue in or near the catalytic site. In the first step, an essential cysteine residue(s) in E1 was treated with TPP plus pyruvate, prior to reaction of excess NEM with the nonessential cysteine residues followed by quenching with dithiothreitol. The reactants were then removed by dialysis, and the residual catalytic activity was determined to be approximately 80% of the original activity. This modified enzyme was then incubated with $^{14}$C]NEM to specifically label any protected cysteine residue(s). The stoichiometry of incorporation of NEM and residual activities were determined as a function of time (Fig. 3). There was a direct correlation between the fractional loss of E1 activity and the NEM incorporation into E1. Ninety percent inactivation of E1 was achieved with the introduction of two mol of $^{14}$C]NEM per mol of E1 tetramer, suggesting that either the $\alpha$ or $\beta$ of E1 subunit contains an essential cysteine residue. This was further confirmed by SDS-gel electrophoresis and autoradiography of the NEM-labeled E1 (Fig. 3, inset). During a 4-min

![Fig. 1. Kinetics of inactivation of E1 by NEM. E1 (3 µM) was incubated with different concentrations of NEM in 20 mM phosphate buffer (pH 7.5) at 30 °C. At the indicated time interval aliquots were taken to measure the remaining PDC activity. The inset shows the concentration dependence of the pseudo first order inactivation rate constant ($k_{app}$) determined from the slope of the inactivation lines for each of the NEM concentrations employed. The concentrations of NEM used were: 0.1 mM (△), 0.2 mM (○), 0.4 mM (▲), and 0.6 mM (▼).](image-url)
incubation the radioactivity appeared only in the α subunit, but upon allowing the alkalization reaction to proceed for 12 min, a small amount of radioactivity also appeared in the β subunit. However, the small level of incorporation into the β subunit did not constitute alklyation of even one site per tetramer, and thus it cannot explain the observed loss of activity.

Identification of Cysteine Residues—To determine the location of [14C]labeled NEM within the protein, radiolabeled E1 was digested with V8 protease. Since it was noted earlier that the β subunit of E1 was resistant to trypsin digestion (Barrera et al., 1972), we have chosen V8 protease to digest E1 because of its high specificity for cleavage of peptide bonds on carboxyl side of glutamic and aspartic acid residues. The resulting peptide mixture was subjected to analysis by reverse phase HPLC. Fig. 4 shows the separation profile for the peptides and the amount of radioactivity associated with each fraction. Radioactivity identified in A (indicated by plus sign) corresponded to fraction 62 in Fig. 4B. No radioactivity was detected in the fractions beyond fraction 80 in Fig. 4B (results not shown).

The NEM-labeled peptide was further analyzed by Edman degradation sequencing procedures along with measuring the radioactivity released following each cycle of degradation (Fig. 5). Radioactivity appeared only in the 16th cycle which corresponded to cysteine. The specificity of this modification is evident because the second cysteine residue (corresponding to cycle 19 in Fig. 5) was not protected by TPP plus pyruvate and hence did not contain radioactivity when E1 was deprotected and labeled with [14C]NEM. Comparison of the observed amino acid sequence (LKADQLYKQKIRF*CHLCD) of bovine E1α peptide with the deduced amino acid sequence of human E1α established that the cysteine equivalent to the cysteine at position 62 of human E1α reacted with NEM following deprotection of the active site. The sequence obtained also shows that there is complete identity between bovine and human proteins in this region. The sequence obtained spans amino acids 47–66 of human E1α (Ho et al., 1989).

DISCUSSION

In the present work, the involvement of cysteine residues in the catalytic function of bovine E1 was indicated by the inactivation of the enzyme by the thiol-specific reagent NEM. Kinetic analysis of the reaction of NEM supports the conclusion that modification of only 1 cysteine/single subunit resulted in the loss of activity. Protection studies with TPP plus pyruvate indicated the involvement of a unique thiol in maintaining the E1α activity. Since TPP plus pyruvate protects the enzyme from NEM inactivation, it is suggested that the essential cysteine is protected by the HE=TPP intermediate. Following deprotection of the active site, 2 cysteine residues/tetramer of E1 are found to be selectively alkylated by [14C]NEM. This result further supports the presence of two active centers per E1 tetramer and that each of these centers has equivalent catalytic efficiency (Khailova and Korochkina, 1982).

The sequence of the peptide generated from bovine E1 containing the labeled cysteine residue is LKADQLYKQKIRF*CHLCD. The complete identity between human and bovine E1α sequences establishes that the modified cysteine residue corresponds to position 62 in the human E1α sequence. The fact that only one cysteine was labeled with [14C]NEM demonstrates a high degree of specificity was achieved. The relatively high rate of enzyme inactivation indicates a high reactivity of Cys-62, suggesting a unique role of Cys-62 in the reaction catalyzed by E1. This cysteiny1 residue is conserved in the aligned amino acid sequences of E1α from the PDC of rat (Matuda et al., 1991), pig (Urata et al., 1991), human (Ho et al., 1989), and yeast (Behal et al., 1989). Among E1 that are αβαβ tetramers, the only known exception to this pattern comes from E1α of Bacillus stearothermophilus (Hawkins et al., 1990) which contains tyrosine at the aligned position. We suggest that this reactive cysteine has an essential role in catalysis. The possibility of a cysteine at the active site was first indicated by spectroscopic data for an acyl intermediate formed by pigeon breast E1 (Khailova et al., 1985). According to the proposed mechanism, the interaction of holo-E1 with pyruvate leads to an intramolecular oxidative transfer of the substrate bound to the protein moiety with the formation of the acetyl-thio enzyme. Frey et al. (1989) have proposed the role of thiol groups in E. coli E1 as chemical catalyst. The formation of the HE=TPP and eventual decomposition of the adduct by E1 involves general acid-base catalysis and the cysteiny1 sulfhydryl could contribute to that catalytic process. The results from another study
Fig. 4. Separation and identification of radiolabeled peptides by HPLC. A, reverse phase HPLC profile of the V8 protease-digested [14C]NEM-labeled E1 showing absorbance at 220 nm versus retention time. The plus sign indicates the position of a radioactive peak. B, distribution of radioactivity in the peptide fractions.

Fig. 5. Amino acid sequence determination of the 14C-radiolabeled peptide. The total number of counts detected in each cycle was plotted against cycle number and the identified amino acids in the peptide are shown by their one-letter codes.

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concerning the E1 of the branched chain keto acid dehydrogenase complex in E. coli also indicated the presence of an essential thiol group at the active site in this related enzyme (Schwartz and Reed, 1970).

In summary, we have identified the essential cysteinyl residue in or near the active site of bovine E1. The identification of the essential cysteine residue represents the first of several other amino acid residues implicated in E1 catalysis. The support for the role of this cysteinyl residue in E1 catalysis will come from future studies involving site-directed mutagenesis and overexpression of mutant mammalian E1.