exposure of new tyrosines parallels the rate of exposure of the C-terminus. This observation is consistent with the notion that tyrosine residues 92 and 25 unfold (Bigelow, 1961; Scott and Scheraga, 1963; Li *et al.*, 1966) before the C-terminal region "melts." In the temperature range in which the C-terminal region unfolds, Tyr-97 becomes exposed to solvent (Li *et al.*, 1966), although the hydroxyl group must remain in an ordered region of the polypeptide because the spectrum of this residue is normalized only in 8 M urea (Bigelow, 1961).

In summary, the C-terminal region of ribonuclease unfolds at higher temperatures than some of the abnormal tyrosine residues begin to normalize, in the reversible thermal denaturation of this protein.

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Added in Proof

In a related paper (A. W. Burgess and H. A. Scheraga, *J. Theor. Biol.*, submitted), a mechanism has been presented for the pathway of unfolding of ribonuclease. In that mechanism, the C-terminal residues were postulated to unfold late in the thermal transition. The present paper constitutes experimental evidence for that aspect of the mechanism.

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Identification of the Lysine Residue Modified during the Activation by Acetimidylation of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: A single amino group in horse liver alcohol dehydrogenase was modified with methyl [^{14}C]acetimidate by a differential labeling procedure. Lysine residues outside the active site were modified with ethyl acetimidate while a lysine residue in the active site was protected by the formation of an enzyme-NAD^+-pyrazole complex. After the protecting reagents were removed, the enzyme was treated with methyl [^{14}C]acetimidate. Enzyme activity was enhanced 13-fold as 1.1 [^{14}C]acetimidyl group was incorporated per active site. A labeled peptide was isolated from a tryptic-chymotryptic digest of the modified enzyme in 35% overall yield. Amino acid composition and sequential Edman degradations identified the peptide as residues 219-229; lysine residue 228 was modified with the radioactive acetimidyl group.

Jörnvall (1973) and Tsai *et al.* (1974) have suggested that reductive methylation of several lysine residues of horse liver alcohol dehydrogenase (EC 1.1.1.1) increases the enzymatic activity, while Plapp *et al.* (1973) showed that amidination of a single residue increases the turnover numbers tenfold. Steady-state and stopped-flow kinetic studies show that the enchanced activity is due to increases in the rate-limiting steps of the forward and reverse reactions, namely the dissociation of the enzyme-coenzyme complexes (Plapp, 1970; Plapp *et al.*, 1973). In order to understand the role of the amino group(s) in the activity of the enzyme we must locate the modified lysine residue(s) in the primary and tertiary structure. In the present work, one lysine residue per active site was differentially amidinated with methyl [^{14}C]acetimidate and identified.

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Experimental Procedure

Crystalline horse liver alcohol dehydrogenase was prepared by a modification of the method of Theorell *et al.* (1966) and assayed as described (Plapp, 1970). Ethyl acetimidate was purchased from Eastman Organic Chemicals; methyl [^{14}C]acetimidate was prepared from [^{14}C]acetonitrile (International Chemical and Nuclear) according to Hunter and Ludwig (1972). The specific activity of the methyl [^{14}C]acetimidate (0.42 mCi/μmol) was corroborated by determining the incorporation of [^{14}C]acetimidate into native alcohol dehydrogenase; 50 of the 60 lysine residues were modified, just as found previously (Plapp *et al.*, 1973). Sequenal-grade phenyl isothiocyanate was purchased from Pierce, fluorescamine from Roche Diagnostics, and trypsin and chymotrypsin from Worthington.

Amino acid compositions were obtained with a narrow-bore, single-column analyzer designed by Liao *et al.* (1973), using fluorescamine for detection (Stein *et al.*, 1973). Hydrolysis times of 22 and 46 hr were used for the estimation of acetimidyllysine (Plapp and Kim, 1974) and isoleucine. Peptides were located by alkaline hydrolysis and ninhydrin analysis (Fruchter and Crestfield, 1965; Moore, 1968). Liquid scintillation counting was performed in 10 ml of Bray's counting solution (Bray, 1960).

Results

Liver alcohol dehydrogenase (100 mg) was partially acetimidylated with unlabeled ethyl acetimidate in the presence of NAD^+ and pyrazole and the excess reagents were removed as described previously (Plapp *et al.*, 1973). The amino groups at the active sites were modified by reaction for 1 hr with 0.1 M methyl [^{14}C]acetimidate in 0.5 M triethanolamine hydrochloride buffer (pH 8.0) at 25°. Enzyme activity increased 13-fold. Incorporation of [^{14}C]acetimidate into partially acetimidylated enzyme indicated that 1.1 ± 0.1 residues was modified per subunit.

The cysteine residues of the acetimidylated enzyme were aminoethylated (Cole, 1967), the protein was digested with trypsin and chymotrypsin, and the labeled peptide was isolated by gel filtration and ion-exchange chromatography as shown in Figure 1A–C. The major radioactive peak from Figure 1A contained 66% of the total radioactivity. Chromatography on Aminex A-5 of the radioactive material in all of the minor peaks from chromatograms such as that shown in Figure 1A resolved a mixture of radioactive peptides; none of these peptides occurred in more than 5% yield. These peptides may have been derived from nonspecific labeling and incomplete proteolysis. The single radioactive peptide purified in Figure 1C represented 35% of the original label. The composition of the peptide was (Asp 2.9, Gly 1.0, Val 1.0, Ile 2.6, Phe 0.9, AILys 2.0[1]). The primary sequence was determined by a modified Edman degradation procedure (Tarr, 1974). The results (Table I) fit the sequence of residues 219–229 (Ile-Ile-Gly-Val-Asp-Ile-Asn-Lys-Asp-Lys-Phe) in the complete sequence determined by Jörnvall (1970). Excluding some radioactivity lost due to extraction of the peptide in the initial cycles of sequential degradation, the radioactive label was found exclusively in AILys-228.

More evidence for the modification of Lys-228 and the exclusion of Lys-226 was obtained from partial acid hydrolysis designed to selectively cleave out the aspartic acid residues. The peptides were chromatographed on Aminex A-5 (Figure 1D). Peak *a* contained (Gly 0.5, Ile 1.0, Phe 1.0, Lys 0.2, AILys 0.7) after 22 hr hydrolysis, but sequential analysis gave only Ile-Ile-Gly. Peaks *b* and *c* had the same amino acid composition (Phe 1.0, Lys 0.2, AILys 0.8). Sequential analysis of these peptides was not performed due to lack of material.

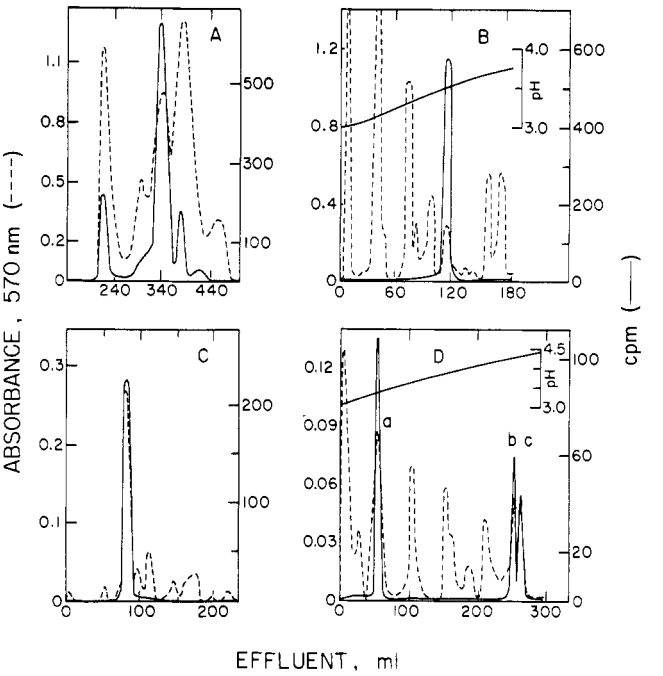

FIGURE 1: Purification of labeled peptide from a proteolytic digest of differentially labeled alcohol dehydrogenase. Trypsin and chymotrypsin (each totaling 1% of the concentration of the protein) were added at 0 and 4 hr; the digestion was carried out for 8 hr at pH 8.0 and 37° in a Radiometer pH-Stat. (A) Separation of tryptic–chymotryptic peptides on a column (2.4 × 93 cm) of Sephadex G-25. The column was developed at 22° with 0.1 M acetic acid at a flow rate of 30 ml/hr. (B) Chromatography of peptides from the major radioactive peak in (A) on Aminex A-5. The column (0.9 × 17 cm) was developed with a 500-ml linear gradient of 0.2 M pyridine (adjusted to pH 3.1 with acetic acid) to 2.0 M pyridine (to pH 5.0 with acetic acid) at 30 ml/hr and 50°. The complete elution pattern is not shown; however, the radioactive peak corresponds to 85% of the total label applied to the column. (C) Elution pattern of the radioactive material from (B) on Bio-Rad AG1-X2. The column (0.9 × 18 cm, through 400 mesh) was developed at 30 ml/hr and 37° with a gradient of decreasing pH (Schroeder, 1967). (D) The labeled peptide from (C) was partially hydrolyzed in 0.03 M HCl (pH 2.9, 110°, 24 hr (Schultz *et al.*, 1962)) and chromatographed on Aminex A-5 as described in (B).

Discussion

A single lysine residue in partially acetimidylated liver alcohol dehydrogenase was specifically labeled with methyl [^{14}C]acetimidate. The peptide containing a majority of the radioactivity was identical with residues 219–229 on the basis of amino acid composition and sequential Edman degradation. Lysine-228 was identified as the residue that is protected by NAD^+ and pyrazole binding and is responsible for the enhancement of enzyme activity upon acetimidylation. The identification of Lys-228 and the elimination of Lys-226 as the modified residue is based upon direct Edman degradation of the labeled peptide and an analysis of the peptides produced by partial acid hydrolysis. In the latter experiments, we expected to find the radioactive peptide AILys-Phe, but the radioactivity was distributed among three peaks (Figure 1D), each containing about equimolar amounts of acetimidyllysine and phenylalanine.

[1] Abbreviation used is: AILys, ϵ-acetimidyllysine.

Table I: Results of Sequential Degradation of the Labeled Peptide.[a]

Cycle	Amino Acid	nmoles	cpm[c]
1	Isoleucine	17.0	87
2	Isoleucine	3.6	46
3	Glycine	11.2	34
4	Valine	6.4	32
5	Aspartic acid	6.4	26
6	Isoleucine	3.2	17
7	Asparagine	*b*	8
8	Al-Lysine		8
9	Aspartic acid		63
10	Al-Lysine		460
11	Phenylalanine		242[d]

[a] For each Edman degradation, 20 nmol of peptide was used. The phenylthiohydantoins from each cycle were either identified by thin-layer chromatography (Amino Acid) or hydrolyzed for 8 hr at 110° in 50 μl of 57% HI in a sealed tube (Smithies *et al.*, 1971) and analyzed as the free amino acids (nmoles). Isoleucine was determined as the sum of isoleucine and alloisoleucine. The results of two analyses are presented: cpm represents about 40% of the extracted phenylthiohydantoins for one analysis and nmoles represents the amino acid recovered after HI hydrolysis of the other. [b] On this particular sequence analysis the peptide was contaminated with salt. As a result, the yields of phenylthiohydantoins were quite low and variable for this sequencing procedure and the degradation appeared to block at Asn-225. [c] Contaminating salt was extracted into acetone for this analysis. Phenylthiohydantoins for cycles 1 through 11 were obtained in good yield and were easily identified by thin-layer chromatography. [d] AlLys was incompletely extracted by this procedure; thus the radioactivity in cycle 11 was due to carry over.

The partial acid hydrolysis apparently produced the diketopiperazine of AlLys-Phe, which hydrolyzed partially and randomly to the dipeptides Phe-AlLys and AlLys-Phe. The diketopiperazine cochromatographed with Ile-Ile-Gly and was resistant to the Edman degradation. Such diketopiperazine formation and hydrolysis under the conditions used for partial acid hydrolysis have been documented also with the similar dipeptide Arg-Phe (Sogin and Plapp, 1974).

Based on results from reductive methylation of the amino groups in the presence and absence of coenzymes and inhibitors that bind to the enzyme, Jörnvall (1973) and Tsai *et al.* (1974) suggested that several lysine residues are involved in the activation of liver alcohol dehydrogenase. Due to the low concentration of formaldehyde in Jörnvall's differential labeling experiments, an average of 10 lysine residues from a total of 60 were modified with unlabeled reagent while the active sites were protected. Subsequent modification with [^{14}C]formaldehyde in the absence of the protecting reagents might be expected to modify about 10 more lysine residues within and without the active sites. In our experiments lysine residues outside the active sites were exhaustively modified with unlabeled ethyl acetimidate, blocking 50 residues and effectively preventing subsequent labeling of these residues with methyl [^{14}C]acetimidate. Incorporation of 1.1 molecules of [^{14}C]acetimidate and recovery of most of the radioactive label in Lys-228 as [^{14}C]acetimidyllysine suggest that only one amino group per active site is involved in the activation. Picolinimidylation of alcohol dehydrogenase that was partially acetimidylated in the presence of NAD^+ and pyrazole also differentially labeled the equivalent of a single lysine residue and activated the enzyme 45-fold (Plapp *et al.*, 1973). The same residue probably is picolinimidylated since the imidoesters react similarly. Lys-228 has also been identified as the residue modified with pyridoxal compounds (Sogin and Plapp, 1974).

The results of chemical and kinetics experiments have led to several proposals for the role of amino groups in the mechanism of alcohol dehydrogenase. The affinity of portions of the NAD^+ molecue (AMP, ADP, adenosine 5′-diphosphoribose) for picolinimidylated enzyme implied that the amino group is near the binding site for the nicotinamide ring (Plapp, 1970). Zoltobrocki *et al.* (1974) suggested that the amino group may be closer to the ethanol binding site, since acetimidylation increased the kinetic constants for ethanol and acetaldehyde without greatly affecting those for the coenzyme. McKinley-McKee and Morris (1972) suggested that the amino group binds the pyrophosphate group of NAD^+.

The identification of Lys-228 as the modified residue allows the amino group to be located in the three-dimensional structure of the enzyme. X-Ray crystallographic analysis of native enzyme and its binary complex with adenosine 5′-diphosphoribose determined the conformation of the polypeptide chain and the coenzyme binding site (Brändén *et al.*, 1973). An electron density map based on 2.4-Å data shows that the amino group of Lys-228 is about 4 Å from the adenosine 3′-hydroxyl and 6 Å from its phosphate, with no direct interaction with the pyrophosphate of the adenosine 5′-diphosphoribose (Eklund *et al.*, 1974; C.-I. Brändén, personal communication). Eklund *et al.* (1974) have suggested that the amino group of Lys-228 forms a hydrogen bond with the carboxyl group of Asp-223, which also forms a hydrogen bond with the adenosine 2′-hydroxyl group. Asp-223 is one of only four invariant residues in homologous positions in the coenzyme binding sites of alcohol, lactate, and glyceraldehyde-3-phosphate dehydrogenases. Modification of Lys-228 in alcohol dehydrogenase may disrupt an interaction critical for the binding of the coenzyme, thereby changing the rates of binding and dissociation. It should be noted, however, that crystals of the enzyme–adenosine 5′-diphosphoribose complex are not isomorphous with crystals of the complexes with NAD^+ or NADH (Zeppezauer *et al.*, 1967). Thus the conformation of the native enzyme and the location of the amino group may change upon binding of the coenzyme.

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An Equilibrium Binding Study of the Interaction of Fructose 6-Phosphate and Fructose 1,6-Bisphosphate with Rabbit Muscle Phosphofructokinase†

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ABSTRACT: Equilibrium binding studies of the interaction of rabbit muscle phosphofructokinase with fructose 6-phosphate and fructose 1,6-bisphosphate have been carried out at 5° in the presence of 1-10 mM potassium phosphate (pH 7.0 and 8.0), 5 mM citrate (pH 7.0), or 0.22 mM adenylyl imidodiphosphate (pH 7.0 and 8.0). The binding isotherms for both fructose 6-phosphate and fructose 1,6-bisphosphate exhibit negative cooperativity at pH 7.0 and 8.0 in the presence of 1-10 mM potassium phosphate at protein concentrations where the enzyme exists as a mixture of dimers and tetramers (pH 7.0) or as tetramers (pH 8.0) and at pH 7.0 in the presence of 5 mM citrate where the enzyme exists primarily as dimers. The enzyme binds 1 mol of either fructose phosphate/mol of enzyme monomer (molecular weight 80,000). When enzyme aggregation states smaller than the tetramer are present, the saturation of the enzyme with either ligand is paralleled by polymerization of the enzyme to tetramer, by an increase in enzymatic activity and by a quenching of the protein fluorescence. At protein concentrations where aggregates higher than the tetramer predominate, the fructose 1,6-bisphosphate binding isotherms are hyperbolic. These results can be quantitatively analyzed in terms of a model in which the dimer is associated with extreme negative cooperativity in binding the ligands, the tetramer is associated with less negative cooperativity, and aggregates larger than the tetramer are associated with little or no cooperativity in the binding process. Phosphate is a competitive inhibitor of the fructose phosphate sites at both pH 7.0 and 8.0, while citrate inhibits binding in a complex, noncompetitive manner. In the presence of the ATP analog adenylyl imidodiphosphate, the enzyme-fructose 6-phosphate binding isotherm is sigmoidal at pH 7.0, but hyperbolic at pH 8.0. The characteristic sigmoidal initial velocity-fructose 6-phosphate isotherms for phosphofructokinase at pH 7.0, therefore, are due to an heterotropic interaction between ATP and fructose 6-phosphate binding sites which alters the homotropic interactions between fructose 6-phosphate binding sites. Thus the homotropic interactions between fructose 6-phosphate binding sites can give rise to positive, negative, or no cooperativity depending upon the pH, the aggregation state of the protein, and the metabolic effectors present. The available data suggest the regulation of phosphofructokinase involves a complex interplay between protein polymerization and homotropic and heterotropic interactions between ligand binding sites.

Rabbit skeletal muscle phosphofructokinase is activated and inhibited by a number of metabolites (Passoneau and Lowry, 1962, 1963). The enzyme exhibits concentration- and pH-dependent aggregation (Pavelich and Hammes, 1973; Aaronson and Frieden, 1972; Leonard and Walker, 1972) as well as sigmoidal initial velocity-fructose 6-phosphate concentration isotherms (Hofer and Pette, 1968). Moreover, a correlation exists between the enzymes' aggregation state and its specific activity in the presence of various activators and inhibitors (Lad *et al.*, 1973). At an enzyme concentration of 0.15 mg/ml and pH 7.0, strong activators such as fructose 1,6-bisphosphate stabilize fully ac-

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