Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Malmström, B. G., & Rosenberg, A. (1959) *Adv. Enzymol. Relat. Areas Mol. Biol. 21*, 131–167.
Mauzerall, D., & Granick, S. (1956) *J. Biol. Chem. 219*, 435–446.
Ramadoss, C. S., Steczko, J., Uhlig, J. W., & Axelrod, B. (1983) *Anal. Biochem. 130*, 481–484.
Ray, M., & Ray, S. (1985) *J. Biol. Chem. 260*, 5913–5918.
Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10–15.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schramm, V. L. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 369–371.
Silver, S., & Kralovich, M. L. (1969) *Biochem. Biophys. Res. Commun. 34*, 640–645.
Silver, S., & Jasper, P. (1977) in *Microorganisms and Minerals* (Weinberg, E. D., Ed.) pp 105–149, Marcel Dekker, New York.
Silver, S., Johnseine, P., & King, K. (1970) *J. Bacteriol. 104*, 1299–1306.
Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273–335.
Williams, R. J. P. (1982) *FEBS Lett. 140*, 3–10.

Selective Carboxymethylation of Cysteine-174 of the $\beta_2\beta_2$ and $\beta_1\beta_1$ Human Liver Alcohol Dehydrogenase Isoenzymes by Iodoacetate†

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ABSTRACT: The $\beta_1\beta_1$ and $\beta_2\beta_2$ human liver alcohol dehydrogenase isoenzymes differ by only one residue at the coenzyme-binding site; Arg-47 in β_1 is replaced by His in the β_2 subunit. Since Arg-47 is thought to facilitate the carboxymethylation of Cys-46 in horse liver alcohol dehydrogenase by binding halo acids in a Michaelis–Menten complex prior to inactivation, the specificity and kinetics of modification of the two human liver $\beta\beta$ isoenzymes with iodoacetate were compared. Both of the $\beta\beta$ isoenzymes were inactivated by treatment with iodo[^{14}C]acetate, and one Cys per subunit was carboxymethylated. Cys-174, which is a ligand to the active-site zinc atom in horse liver alcohol dehydrogenase, was selectively carboxymethylated in each of the human $\beta\beta$ isoenzymes; less than 15% of the iodo[^{14}C]acetate incorporated into the enzyme appeared in Cys-46. Therefore, the three-dimensional structure of the basic amino acids in the anion-binding site of the human $\beta\beta$ isoenzymes appears to be different from that of horse liver alcohol dehydrogenase. The kinetics of alkylation are consistent with the formation of a Michaelis–Menten complex before inactivation of the isoenzymes. The average K_i values for iodoacetate were 10 and 16 mM for $\beta_1\beta_1$ and $\beta_2\beta_2$, respectively, and maximal rate constants for inactivation were 0.22 and 0.17 min^{-1}, respectively. From these data, it can be concluded that there is a relatively minor effect of the substitution of His for Arg at position 47 on the kinetics of inactivation.

A variety of active-site-directed chemical modifying reagents have been used to investigate the structure and mechanism of horse liver alcohol dehydrogenase. The halo acids iodoacetate and 3-bromopropionate have been shown to selectively alkylate the Cys-46 and Cys-174 residues, respectively, both of which are active-site zinc ligands (Li & Vallee, 1964; Harris, 1964; Chadha & Plapp, 1984). Enzyme alkylated with iodoacetate retains less than 5% of the original catalytic activity (Reynolds & McKinley-McKee, 1970). The kinetics of inactivation by these reagents indicate that a high-affinity, reversible enzyme–inhibitor Michaelis–Menten complex is formed prior to covalent modification (Reynolds & McKinley–McKee, 1969; Chadha & Plapp, 1984). NAD^+ and NADH competitively inhibit inactivation by iodoacetate. On the basis of X-ray crystallographic studies of the Cys-46 carboxymethylated enzyme (Zeppezauer et al., 1975) and chemical modification studies of Arg residues (Lange et al., 1975), it has been suggested that iodoacetate binds to Arg-47 prior to alkylation of Cys-46 to form the reversible Michaelis–Menten complex. The alkylation reaction of Cys-46 in horse liver alcohol dehydrogenase by iodoacetate has been used to investigate the specificity and affinity of anions, such as NAD(H) analogues, $Pt(CN)^{4-}$, phosphate, and halides, for the anion-binding site (Reynolds & McKinley-McKee, 1969; Reynolds et al., 1970; Zeppezauer et al., 1975; Dahl & McKinley-McKee, 1980).

Human liver alcohol dehydrogenase exists in multiple molecular forms, and a genetic model accounts for this multiplicity (Smith et al., 1971; Bosron & Li, 1981; Vallee & Bazzone, 1983). The model assumes that there are five separate gene loci with polymorphism at two loci. The two alloenzymes that are produced at ADH_2, $\beta_1\beta_1$ and $\beta_2\beta_2$, exhibit different pH optima for ethanol oxidation (10.5 and 8.5, respectively), V_{max} values for ethanol oxidation at pH 7.5 (9.2 and 400 min^{-1}), and K_m values for NAD^+ (7.4 and 180 μM). Since the β_1 subunit predominates in Caucasians while the β_2 subunit occurs most frequently in Orientals (Yin et al., 1984a), it was suggested that such polymorphism of alcohol dehydrogenase isoenzymes may contribute to differences in the alcohol elimination rate between these racial groups (Stamatoyannopoulos et al., 1975).

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Yoshida et al. (1981) observed that the $\beta_1\beta_1$ and $\beta_2\beta_2$ human liver alcohol dehydrogenase isoenzymes differed in their sensitivity to inactivation by iodoacetate; $\beta_1\beta_1$ was inactivated by iodoacetate while $\beta_2\beta_2$ was resistant to inactivation. They ascribed this difference in iodoacetate sensitivity of the $\beta_2\beta_2$ isoenzyme to the substitution of the susceptible active-site Cys-46 residue in $\beta_2\beta_2$ by His. Jörnvall et al. (1984) reexamined the sequence of the human liver β_1 and β_2 subunits and found that Cys-46 was conserved in both isoenzymes but that Arg-47 in β_1 was replaced by His in β_2. Subsequent studies of the β_1 cDNA sequence have confirmed the assignment of Cys-46 and Arg-47 in this subunit (Ikuta et al., 1985). Since the affinity of $\beta_2\beta_2$ for NAD^+ and NADH is substantially less than that of $\beta_1\beta_1$ (Yin et al., 1984b), and the Cl^- ion does not affect the activity of $\beta_2\beta_2$ while it stimulates that of $\beta_1\beta_1$ (Bühler & VonWartburg, 1984; Bosron et al., 1985), it appears that the substitution of His-47 for Arg-47 substantially alters the properties of the anion-binding site of the enzyme. Accordingly, we have examined the kinetics of inactivation of the $\beta_2\beta_2$ and $\beta_1\beta_1$ human liver alcohol dehydrogenase isoenzymes by iodoacetate (Bosron et al., 1985) and the position of the residues in the β_2 and β_1 sequences that are alkylated.

Materials and Methods

Purification of ββ Isoenzymes. $\beta_2\beta_2$ and $\beta_1\beta_1$ were isolated from livers with the ADH_2 2-2, ADH_3 1-1 and ADH_2 1-1, ADH_3 1-1 phenotypes, respectively. The two isoenzymes were purified by homogeneity by ion-exchange and affinity chromatography (Bosron et al., 1983; Yin et al., 1984a). The purified isoenzymes exhibited single protein and activity bands on starch gel electrophoresis and agarose isoelectric focusing (Yin et al., 1984b) and a single protein band of approximately 40 000 daltons on sodium dodecyl sulfate (SDS)[1]–polyacrylamide gel electrophoresis after silver staining (Yin et al., 1984b; Wray et al., 1981). For chemical modification studies, $\beta_1\beta_1$ and $\beta_2\beta_2$ were gel filtered on Bio-Gel P-6 (Bio-Rad, Richmond, CA) in 10 mM NaP_i, pH 7.5. For peptide mapping and sequence studies, the isoenzymes were dialyzed against 0.1 M NH_4HCO_3 and lyophilized.

Kinetic Studies of Iodoacetate Inactivation. The purified ββ isoenzymes were diluted to 7–10 μM in 0.1 M NaP_i containing varying concentrations of iodoacetate (Sigma, St. Louis, MO) at pH 7.5, 25 °C. Aliquots were removed at specific times between 10 and 120 min, and the modified and unmodified control enzyme samples were assayed spectrophotometrically at 340 nm and 25 °C in 0.1 M glycine–NaOH, pH 10.0, with 33 mM ethanol and 2.4 mM NAD^+ (grade I, Boehringer-Mannheim, Indianapolis, IN) as substrates. The apparent first-order rate constant for inactivation, k_3, was determined by plotting the log of the modified enzyme activity divided by control activity vs. time. The formation of a Michaelis–Menten complex with iodoacetate was verified by plotting 1/inactivation rate vs. 1/iodoacetate concentration (Reynolds & McKinley-McKee, 1969). The dissociation constant for iodoacetate, K_i, and the dissociation constants for the competitive inhibitors of inactivation, NAD^+ and NADH, were calculated with the COMP program of Cleland (1979).

Amino Acid Analysis, Peptide Mapping, and Sequencing of Carboxymethylated ββ Isoenzymes. The purified ββ isoenzymes (10 μM) were incubated with a 1000-fold molar excess of iodo[2-^{14}C]acetate (0.5 Ci/μmol) (Amersham, Arlington Heights, IL) at 25 °C for 100 min. The reaction was terminated by passage of the solution over Bio-Gel P-6 equilibrated with 10 mM NaP_i, pH 7.5, to separate excess iodoacetate from the enzyme. The protein was dialyzed against 0.1 M NH_4HCO_3 at 4 °C overnight and lyophilized. The labeled enzyme was reduced with dithiothreitol in 6 M guanidine hydrochloride and exhaustively carboxymethylated with unlabeled iodoacetate as described by Crabb et al., (1983). The incorporation of [^{14}C]carboxymethyl groups per mole of ADH was determined by liquid scintillation counting in Amersham PCS liquid fluor. [^{14}C]Toluene (New England Nuclear, Boston, MA) was added to determine counting efficiency. The stoichiometry of incorporation of carboxymethyl groups was based on the measurement of ββ protein concentration by the method of Lowry et al. (1951).

For studies of the specific amino acids that incorporate [^{14}C]carboxymethyl groups, enzyme was hydrolyzed in 6 N HCl and 5% phenol under reduced pressure at 110 °C for 22 h. The amino acid composition was determined on a Beckman 119 CL amino acid analyzer. In a duplicate run, the effluent from the analyzer was collected, and the incorporation of [^{14}C]carboxymethyl groups into amino acids was determined by scintillation counting.

For sequence analysis, carboxymethylated alcohol dehydrogenase (4–5 mg or 100–125 nmol of subunit at a concentration of 1 mg/mL) was dissolved in 70% formic acid. A 20-fold excess of CNBr by weight was added, the sample was incubated under N_2 overnight at 25 °C in the dark, and it was lyophilized. Urea was added to the peptides to 8 M, and they were gel filtered on a 2.5 × 100 cm column of Sephadex G-75 (Pharmacia, Piscataway, NJ) in 30% acetic acid (Baker Ultrex grade, Philadelphia, PA). The absorbance at 280 nm and the incorporation of [^{14}C]carboxymethyl groups were monitored in the effluent. The fractions containing the high molecular weight radioactive peptide peak were pooled and lyophilized.

The amino acid sequence of part of the largest CNBr fragment, residues 41–209 (Jörnvall et al., 1984), was determined with a Beckman 890C liquid-phase sequenator using the 0.1 M Quadrol peptide program (121078; Dwulet & Benson, 1983). To all samples was added 3 mg of polybrene to prevent excessive peptide extraction from the reaction cup. Before degradation was begun, the peptide and polybrene were subjected to one cycle in the sequenator to which no phenyl isothiocyanate was added. the PTH-amino acid derivatives were identified by their retention time relative to a norleucine internal standard after reversed-phase HPLC on an Altex Ultrasphere C-18 column (4.6 × 250 mm; Dwulet & Benson, 1983). The amount of peptide sequenced was estimated from the total radioactivity divided by the initial specific radioactivity of the carboxymethylated subunits. The recovery of amino acids sequenced was estimated by comparison of the absorbance of PTH-amino acids eluted by HPLC with that of standards. Samples of the organic and aqueous phases were examined for [^{14}C]carboxymethyl incorporation by liquid scintillation counting.

Approximately 20% of the large CNBr fragment obtained by G-75 Sephadex chromatography, which had not been sequenced and contained both Cys-46 and Cys-174, was cleaved with 2% TPCK–trypsin as described by Rex et al. (1984) and lyophilized. Tryptic peptides were dissolved in 0.1% trifluoroacetic acid (Pierce, Rockford, IL), applied to a 4.6 × 250 mm Synchrom RP-8 HPLC column, and eluted with a linear gradient of 0–60% CH_3CN in 0.1% trifluoroacetic acid. Peptides in the effluent were monitored at 215 nm, and [^{14}C]carboxymethyl groups were determined by liquid scin-

[1] Abbreviations: PTH-amino acid, phenylthiohydantoin derivative of an amino acid; HPLC, high-performance liquid chromatography; TPCK, 1-chloro-3-(tosylamido)-4-phenylbutan-2-one; SDS, sodium dodecyl sulfate.

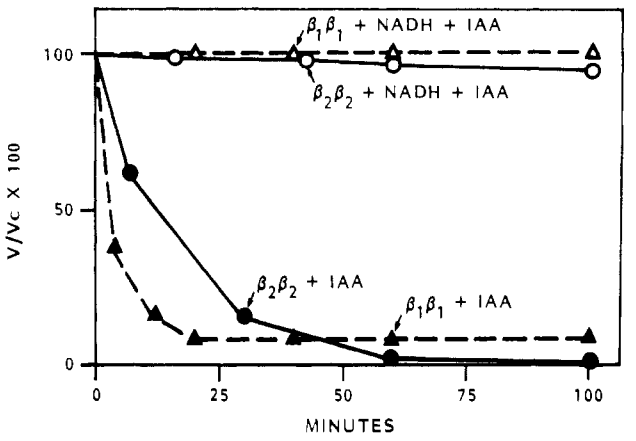

FIGURE 1: Time-dependent inactivation of $\beta\beta$ isoenzymes by iodoacetate and protection by NADH. Fractional residual activity, V/V_c, is plotted against the time of incubation. The isoenzymes, 10 μM, were incubated with 10 mM iodoacetate (IAA) in 0.1 M NaP_i, pH 7.5, 25 °C, in the absence or presence of NADH (5 mM for $\beta_2\beta_2$ and 1 mM for $\beta_1\beta_1$). Aliquots were removed at the indicated times, and ethanol oxidizing activity was assayed as described under Materials and Methods.

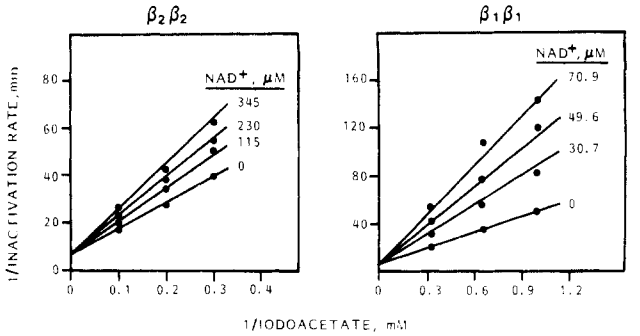

FIGURE 2: Kinetics of inactivation of $\beta\beta$ isoenzymes by iodoacetate and protection by NAD^+. $\beta_2\beta_2$ (10 μM) and $\beta_1\beta_1$ (10 μM) were preincubated with the indicated concentration of NAD^+ in 10 mM NaP_i, pH 7.5, 25 °C, for 30 min. The indicated concentration of iodoacetate was added; aliquots were removed at 10-, 20-, 30-, 45-, 60-, and 90-min intervals and assayed for enzymatic activity at pH 10.0 as described under Materials and Methods. The pseudo-first-order rate constants of inactivation were evaluated by plotting log activity vs. time (not shown), and the reciprocals of these values were plotted against the reciprocal of the iodoacetate concentration. Values for K_i and the maximal rate of inactivation were evaluated by using the COMP program of Cleland (1979).

tillation counting. The labeled peptide was sequenced as described above.

RESULTS

Inactivation of $\beta\beta$ Isoenzymes by Iodoacetate. Both $\beta_1\beta_1$ and $\beta_2\beta_2$ (10 μM) were rapidly inactivated by 10 mM iodoacetate at pH 7.5 and 25 °C. As shown in Figure 1, the rate of inactivation of $\beta_2\beta_2$ was slower than that of $\beta_1\beta_1$. $\beta_2\beta_2$ was inactivated to less than 1% of the initial activity at 100 min, while $\beta_1\beta_1$ reached a minimum of about 10% of the initial activity. However, this preparation of $\beta_1\beta_1$ contained trace amounts of tightly bound NADH. When bound NADH was converted to NAD^+ with 1 mM acetaldehyde in 0.1 M NaP_i, pH 7.5, and removed by gel filtration (Crabb et al., 1983), $\beta_1\beta_1$ exhibited less than 2% of the initial activity after a 100-min incubation. NADH (1 and 5 mM) protected both isoenzymes against inactivation by iodoacetate (Figure 1). NAD^+ was less effective than NADH in protecting the $\beta\beta$ isoenzymes against inactivation. This is consistent with the observation that the K_i values of the isoenzymes are greater for NAD^+ than for NADH at pH 7.5 (Yin et al., 1984b).

During the 100-min reaction period, 1.02 and 0.99 carboxymethyl groups were incorporated per β_2 and β_1 subunit, respectively, as measured with iodo[2-^{14}C]acetate. The carboxymethylated isoenzymes were subjected to amino acid analysis, and radioactivity was recovered solely under the *S*-(carboxymethyl)cysteine peak. In agreement with the radioactivity data, the number of (carboxymethyl)cysteine residues formed were 1.29 and 1.34 per subunit of β_2 and β_1, respectively. In the presence of NADH, only 0.05 and 0.10 sulfhydryl groups were carboxymethylated in β_1 and β_2, respectively.

The rate of inactivation of the two isoenzymes was examined at different iodoacetate concentrations. The kinetics of inactivation were first order with respect to enzyme. Plots of 1/inactivation rate vs. 1/[iodoacetate] were consistent with the formation of a Michaelis–Menten complex (Figure 2). In duplicate experiments, the dissociation constants, K_i, of $\beta_2\beta_2$ for iodoacetate were 15 ± 2 and 16 ± 1 mM; values for $\beta_1\beta_1$ were significantly less, 8.5 ± 1.4 and 10.9 ± 2.4 mM. Both NAD^+ (Figure 2) and NADH were competitive inhibitors of the iodoacetate-dependent inactivation. The slope inhibition constant of $\beta_2\beta_2$ for NAD^+, 470 ± 30 μM (Figure 2), agreed

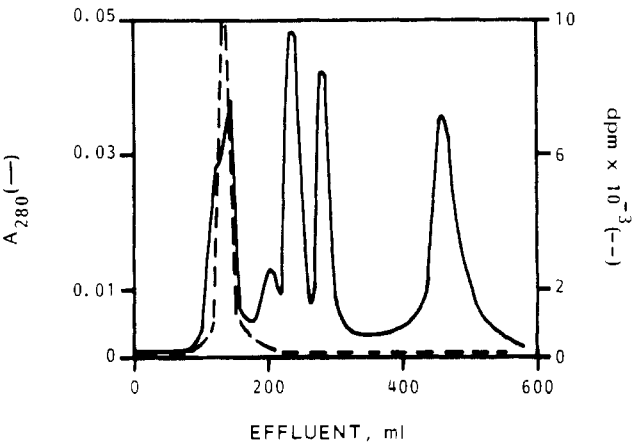

FIGURE 3: Gel filtration of ^{14}C-carboxymethylated $\beta_2\beta_2$ CNBr peptides. $\beta_2\beta_2$, approximately 100 nmol of subunit, was selectively carboxymethylated with iodo[^{14}C]acetate, reduced with dithiothreitol, and exhaustively carboxymethylated with unlabeled iodoacetate as described under Materials and Methods. The inactive isoenzyme was cleaved with CNBr and gel filtered on Sephadex G-75 in 30% acetic acid as described under Materials and Methods. The solid line represents the elution profile of peptides monitored at 280 nm, and the dashed line indicates the elution of peptide(s) specifically carboxymethylated with labeled iodoacetate.

well with the K_{ia} value reported by Yin et al. (1984b), 340 μM. The slope inhibition constant of $\beta_1\beta_1$, 35 ± 2 μM (Figure 2), was less than the K_{ia} value for NAD^+ determined for this isoenzyme, 90 μM. The maximal rate constant for inactivation, k_3, calculated from the data in Figure 2 was 0.17 ± 0.02 min^{-1} for $\beta_2\beta_2$ and 0.22 ± 0.05 min^{-1} for $\beta_1\beta_1$. The pseudo-bimolecular rate constant for inactivation by iodoacetate, k_3/K_i, was 11 M^{-1} min^{-1} for $\beta_2\beta_2$ and 23 M^{-1} min^{-1} for $\beta_1\beta_1$.

Identification of Amino Acid Residues Carboxymethylated by Iodoacetate in $\beta_1\beta_1$ and $\beta_2\beta_2$. The active-site-^{14}C-carboxymethylated, inactive isoenzymes were exhaustively carboxymethylated with unlabeled iodoacetate, cleaved with CNBr, and gel filtered on Sephadex G-75. The elution profile of the CNBr fragments of $\beta_2\beta_2$, shown in Figure 3, is similar to that reported by Jörnvall et al. (1984) on Sephadex G-50, except that a shoulder on the high molecular weight peak was resolved in this study. ([^{14}C]Carboxymethyl)cysteine appeared only in the high molecular weight peak between 115 and 155 mL of effluent; 81% of the counts applied were recovered in

Table I: Sequence and [^{14}C]Carboxymethyl Incorporation into Amino Acids of the Large CNBr Fragment of β_2 and β_1[a]

	β_2 subunit		β_1 subunit	
cycle	residue (nmol)	^{14}C dpm	residue (nmol)	^{14}C dpm
1	Val (7)	13	Val (6)	5
2	Ala (11)	16	Ala (6)	6
3	Val (11)	6	Val (6)	6
4	Gly (11)	9	Gly (4)	8
5	Ile (9)	15	Ile (5)	14
6	Cys (10)	667	Cys (4)	665
7	His (2)	86	Arg (3)	151
8	Thr (4)	22	Thr (3)	47
		10830[b]		9330[b]

[a] Approximately 20% of the total radioactivities obtained in the large CNBr fragment of β_2 (Figure 3), 15400 dpm, and β_1, 11000 dpm, were sequenced as described under Materials and Methods. On the basis of the initial specific activity of carboxymethylation for β_2 and β_1, 1200 dpm/nmol of subunit, approximately 13 and 9 nmol of fragment were sequenced, respectively. The recovery of PTH-amino acids and counts are indicated for each cycle. Cycles 1–8 correspond to residues 41–48 of the β subunits. The unsequenced fraction, containing about 91% of the counts, was the residue remaining in the cup. [b] Unsequenced.

this peak. The elution profiles of two different preparations of $\beta_1\beta_1$ and one additional preparation of $\beta_2\beta_2$ were similar to that shown in Figure 3.

Even though the large CNBr fragment was not homogeneous, previous sequence analysis had shown no N-terminal heterogeneity (Jörnvall et al., 1984; Hempel et al., 1984). The N-terminal amino acid of alcohol dehydrogenase, Ser, is blocked (Hempel et al., 1984). As shown in Table I, the sequence of the first eight residues of the large CNBr peptide of β_1 and β_2 was identical, except for the substitution of His in cycle 7 of β_2 (amino acid position 47) for Arg at the same position in β_1. The substitution of His-47 for Arg-47 is the only difference between these two alloenzymes (Jörnvall et al., 1984). Only 5–7% of the recovered ([^{14}C]carboxymethyl)-cysteine counts appeared in cycle 6, Cys-46, for β_2 and two different preparations of β_1. The majority of the counts, 90–95% in Table I, appeared in the unsequenced portion of the peptide. However, this fraction might have also contained a portion of the CNBr fragment that had not been coupled to the sequencer resin. To correct for the amount of label appearing in cycle 6 for the portion of the fragment that was not coupled, the recovery of (carboxymethyl)cysteine in cycle 6 was calculated. As shown in Table I, this was 10/13 nmol or 77% and 4/9 nmol or 44% for β_2 and β_1, respectively, and these values are similar to the average recovery of the first six residues sequenced for each subunit. On the basis of these recoveries, the maximum amount of labeled Cys-46 that could appear in both the sequenced and unsequenced portion of this peptide would be 8% for β_2 and 14% for β_1.

To ascertain more directly whether Cys-46 or another Cys residue in the large CNBr fragment was selectively modified, approximately 20 nmol of the large molecular weight CNBr peptide was cleaved with trypsin, and the tryptic peptides were separated by reversed-phase HPLC. According to Jörnvall et al. (1984), this fragment contains residues 41–209; therefore, it contains both Cys-46 and Cys-174. The elution profile for the β_2 peptides is shown in Figure 4. The elution profile for a second preparation of β_2 and one preparation of β_1 was similar to that shown in Figure 4. One major tryptic peptide of β_2 and β_1 contained the majority of the [^{14}C]carboxymethyl counts, 65% and 71%, respectively, and its elution position was similar for both subunits, about 46 min. For β_1 but not β_2, a second tryptic peptide containing 7% of the recovered radioactivity eluted at about 21 min. The remainder of the

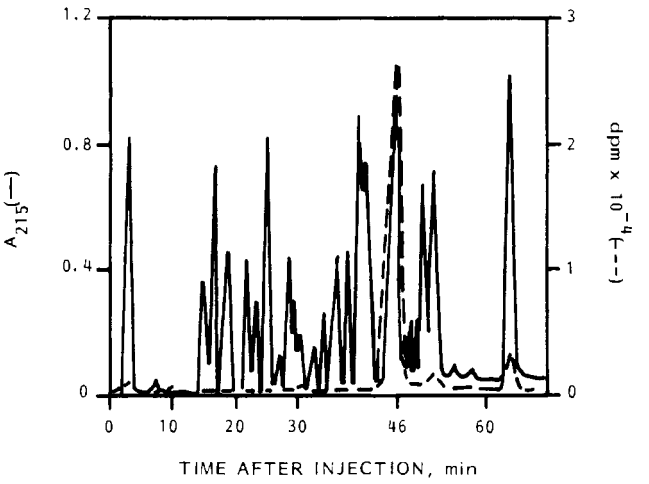

FIGURE 4: Reversed-phase HPLC of tryptic peptides of the large CNBr peptide of ^{14}C-carboxymethylated $\beta_2\beta_2$. Approximately 20 nmol of the large CNBr peptide of β_2 shown in Figure 3 was cleaved with trypsin, applied to a RP-8 HPLC column, and eluted with a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid as described under Materials and Methods. The solid line represents the elution profile of tryptic peptides monitored at 215 nm, and the dashed line indicates the elution of peptides specifically carboxymethylated with labeled iodoacetate.

Table II: Sequence and [^{14}C]Carboxymethyl Incorporation into Amino Acids in a Tryptic Peptide of β_2 and β_1[a]

	β_2 subunit		β_1 subunit	
cycle	residue (nmol)	^{14}C dpm	residue (nmol)	^{14}C dpm
1	Val (9)	9	Val (6)	10
2	Cys (4)	86	Cys (3)	36
3	Leu (5)	72	Leu (6)	40
4	Ile (5)	40	Ile (5)	38
5	Gly (4)	37	Gly (3)	35
6	Cys (4)	4842	Cys (3)	2573
7	Gly (3)	293	Gly (3)	188
8	Phe (5)	58	Phe (3)	55
9	Ser (3)			
10	Thr (1)			
11	Gly (5)			
12	Tyr (3)			
13	Gly (4)			
14	Ser (2)			
15	Ala (3)			
16	Val (4)			
		837[b]		491[b]

[a] A portion of the large CNBr fragment of β_2 and β_1 which had not been sequenced (Table I) and contained both Cys-46 and Cys-174 was cleaved with trypsin, and peptides were separated by HPLC as described under Materials and Methods and Figure 4. The major ^{14}C-carboxymethylated tryptic peptide shown in Figure 4 was sequenced by procedures described under Materials and Methods and in Table I. Cycles 1–16 correspond to residues 169–184 of the β subunits. [b] Unsequenced.

counts were distributed throughout the column effluent. The fraction containing the major radioactive peptide eluting at about 46 min was sequenced. The first 16 residues of the β_2 peptide and the first 8 residues of the β_1 peptide, shown in Table II, corresponded to residues 169–184 and 169–176 of these subunits (Hempel et al., 1984; Jörnvall et al., 1984). The recovery of [^{14}C]carboxymethyl counts in the first eight residues of this tryptic peptide from both β_2 and β_1 is shown in Table II. For the two preparations of β_2, 77% and 85% of the dpm recovered were found in cycle 6 which corresponds to Cys-174 (Table II), and for β_1, 74% of the dpm were found in the Cys-174 cycle. Less than 2% of the counts appeared in cycle 2 which corresponds to Cys-170. The β_1 tryptic peptide containing 7% of the recovered radioactivity was also

sequenced. The results indicate that it was a mixture of two peptides corresponding to residues 41–47, which includes Cys-46, and residues 160–168, which includes no Cys residue.

Discussion

Initial studies of inactivation of human liver alcohol dehydrogenase by iodoacetate indicated that there was a substantial difference in the sensitivity of $\beta_2\beta_2$, the Oriental variant of the $\beta\beta$ isoenzyme, as compared with $\beta_1\beta_1$, the form that appears in the majority of Caucasians. It was reported that $\beta_2\beta_2$ retained about 80% of its activity when treated with 0.5 mM iodoacetate at pH 7.5 for 120 min, while $\beta_1\beta_1$ was completely inactivated (Yoshida et al., 1981). The data seemed to support the first sequence analysis of the β_2 and β_1 subunits which concluded that the active-site Cys of β_1 was replaced by His (Yoshida et al., 1981), thereby precluding the alkylation and inactivation of $\beta_2\beta_2$. However, as shown by the data in Figure 1, both $\beta_2\beta_2$ and $\beta_1\beta_1$ can be inactivated by exposure to a higher concentration of iodoacetate, 10 mM at pH 7.5, with the incorporation of approximately one carboxymethyl group in Cys per subunit. Only the kinetics of inactivation differ for the two isoenzymes. As has been observed with horse liver alcohol dehydrogenase (Reynolds & McKinlee-McKee, 1969), iodoacetate forms a Michaelis–Menten complex with both $\beta_2\beta_2$ and $\beta_1\beta_1$ before modifying a Cys residue (Figure 2). The inactivation of the human liver isoenzymes obeys Michaelis–Menten kinetics. There is a relatively small but significant difference between the affinities of $\beta_2\beta_2$ and $\beta_1\beta_1$ for iodoacetate; average K_i values are 16 and 10 mM, respectively. This results in a 2-fold difference in the pseudo-bimolecular rate constant for inactivation, 11 M^{-1} min^{-1} for $\beta_2\beta_2$ and 23 M^{-1} min^{-1} for $\beta_1\beta_1$, and this appears to account for the difference in inactivation rate of the two isoenzymes shown in Figure 1.

Recent sequence analysis of β_1 and β_2 has shown that Cys-46, a zinc ligand for horse liver alcohol dehydrogenase, is conserved in both isoenzymes and that the two isoenzymes differ by only one amino acid, the substitution of Arg-47 in β_1 by His in β_2 (Jörnvall et al., 1984; Table I). Since the K_m and K_i values for NAD^+ and NADH for $\beta_2\beta_2$ are substantially higher than the respective values for $\beta_1\beta_1$ (Yin et al., 1984b), and $\beta_1\beta_1$ activity is increased by Cl^- but $\beta_2\beta_2$ is unaffected (Bühler & VonWartburg, 1984; Bosron et al., 1985), it was proposed that the substitution of His for Arg at position 47 alters the relative affinity of these isoenzymes for anions at the active site.

Horse liver alcohol dehydrogenase, which also has Arg at position 47, is inactivated by iodoacetate, and the neighboring Cys-46 residue, a ligand to the active-site zinc atom, is stoichiometrically alkylated (Li & Vallee, 1964, 1965). On the basis of chemical modification of Arg and X-ray crystallographic studies (Lange et al., 1975; Zeppezauer et al., 1975), it was proposed that the anion iodoacetate binds to the guanidino group of the Arg-47 residue in a Michaelis–Menten complex and the adjacent Cys-46 residue is specifically alkylated. By analogy to horse liver alcohol dehydrogenase, it was initially assumed that Cys-46 of the human liver $\beta_1\beta_1$ isoenzyme would also be specifically alkylated by iodoacetate (Yoshida et al., 1981; Bosron et al., 1985). The affinity of $\beta_1\beta_1$ for iodoacetate, average K_i = 10 mM, is similar to that of horse liver alcohol dehydrogenase under the same experimental conditions, K_i = 6 mM (S.-J. Yin, unpublished results). The tryptic peptide HPLC map of the large CNBr fragment of β_1, which contains two of the putative active-site zinc ligands, Cys-46 and Cys-174, shows that only one peptide contains the majority of the label after iodo[^{14}C]acetate treatment. The sequence of this peptide indicates that it corresponds to residues 169–188 (Hempel et al., 1984) and that it is not contaminated with other peptides. The majority of the radioactivity appears in Cys-174 (Table II), and only 7% of the radioactivity appears in a peptide containing Cys-46. The data indicate that Cys-174, not Cys-46, is selectively alkylated in the human liver $\beta_1\beta_1$ isoenzyme. This difference in specificity of alkylation by iodoacetate between the horse and human liver alcohol dehydrogenases suggests that the three-dimensional structure of basic amino acids in the anion-binding site of the human liver enzyme may be different from that of the horse liver enzyme, even through they both contain Arg at position 47 and there is 87% sequence homology between the two dehydrogenases (Hempel et al., 1984; Ikuta et al., 1985). The data emphasize the need for detailed structural characterization in chemical modification studies of enzymes from different species even when there is a high degree of sequence homology between the variant forms being examined.

The residue carboxymethylated in $\beta_2\beta_2$ is the same as that in $\beta_1\beta_1$, Cys-174 (Table II). While the substitution of Arg-47 in β_1 by His in β_2 clearly affects the catalytic properties of these isoenzymes (Yin et al., 1985b), there is little effect of this substitution on the specificity or kinetics of alkylation of the $\beta\beta$ isoenzymes by iodoacetate. It is clear that the structure of the human liver $\beta_2\beta_2$ and $\beta_1\beta_1$ anion-binding sites needs to be examined further to identify the basic residues responsible for the formation of the Michaelis–Menten complex and the specific alkylation of Cys-174.

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Registry No. ADH, 9031-72-5; IAA, 144-48-9; NAD, 53-84-9; NADH, 58-68-4; L-Arg, 74-79-3; L-His, 71-00-1; L-Cys, 52-90-4.

References

Bosron, W. F., & Li, T.-K. (1981) *Semin. Liver Dis. 1*, 179–188.

Bosron, W. F., Magnes, L. J., & Li, T.-K. (1983) *Biochemistry 22*, 1852–1857.

Bosron, W. F., Yin, S.-J., & Li, T.-K. (1985) *Enzymology of Carbonyl Metabolism 2: Aldehyde Dehydrogenase, Aldo-Keto Reductase, and Alcohol Dehydrogenase*, pp 193–206, Alan R. Liss, New York.

Bühler, R., & VonWartburg, J.-P. (1984) *FEBS Lett. 178*, 249–252.

Chadha, V. K., & Plapp, B. V. (1984) *Biochemistry 23*, 216–221.

Cleland, W. W. (1979) *Methods Enzymol. 63A*, 103–138.

Crabb, D. W., Bosron, W. F., & Li, T.-K. (1983) *Arch. Biochem. Biophys. 224*, 299–309.

Dahl, K. H., & McKinley-McKee, J. S. (1980) *Eur. J. Biochem. 103*, 47–51.

Dwulet, F. E., & Benson, M. D. (1983) *Biochem. Biophys. Res. Commun. 114*, 657–662.

Harris, J. I. (1964) *Nature (London) 203*, 30–34.

Hemple, J., Bühler, R., Kaiser, R., Holmquist, B., De Zalenski, C., VonWartburg, J.-P., Vallee, B., & Jörnvall, H. (1984) *Eur. J. Biochem. 145*, 437–445.

Ikuta, T., Fujiyoshi, T., Kurachi, K., & Yoshida, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2703–2707.

Jörnvall, H., Hempel, J., Vallee, B. L., Bosron, W. F., & Li, T.-K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3024–3028.

Lange, L. G., Riordan, J. F., Vallee, B. L., & Brändén, C. I. (1975) *Biochemistry 14*, 3497–3502.

Li, T.-K., & Vallee, B. L. (1964) *Biochemistry 3*, 869–873.

Li, T.-K., & Vallee, B. L. (1965) *Biochemistry 4*, 1195–1202.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Rex, D. K., Bosron, W. F., & Li, T.-K. (1984) *Biochem. Genet. 22*, 115–124.
Reynolds, C. H., & McKinley-McKee, J. S. (1969) *Eur. J. Biochem. 10*, 474–478.
Reynolds, C. H., & McKinley-McKee, J. S. (1970) *Biochem. J. 119*, 801–802.
Reynolds, C. H., Morris, D. L., & McKinley-McKee, J. S. (1970) *Eur. J. Biochem. 14*, 14–26.
Smith, M., Hopkinson, D. A., & Harris, H. (1971) *Ann. Hum. Genet. 34*, 251–271.
Stamatoyannopoulos, G., Chen, S.-H., & Fukuk, M. (1975) *Am. J. Hum. Genet. 27*, 789–796.
Vallee, B. L., & Bazzone, T. J. (1983) *Isoenzymes: Current Topics in Biological and Medical Research* (Rattazzi, M. C., Scandalios, J. G., & Whitt, G. S., Eds.) Vol. 8, pp 219–244, Alan R. Liss, New York.
Wray, W., Boulikas, T., Wray, V., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yin, S.-J., Bosron, W. F., Li, T.-K., Ohnishi, K., Okuda, K., Ishii, H., & Tsuchiya, M. (1984a) *Biochem. Genet. 22*, 169–180.
Yin, S.-J., Bosron, W. F., Magnes, L. J., & Li, T.-K. (1984b) *Biochemistry 23*, 5847–5853.
Yoshida, A., Impraim, C. C., & Huang, I.-Y. (1981) *J. Biol. Chem. 256*, 12430–12436.
Zeppezauer, E., Jörnvall, H., & Ohlsson, I. (1975) *Eur. J. Biochem. 58*, 95–104.

Use of Binding Energy in Catalysis Analyzed by Mutagenesis of the Tyrosyl-tRNA Synthetase†

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ABSTRACT: The utilization of enzyme–substrate binding energy in catalysis has been investigated by experiments on mutant tyrosyl-tRNA synthetases that have been generated by site-directed mutagenesis. The mutants are poorer enzymes because they lack side chains that form hydrogen bonds with ATP and tyrosine during stages of the reaction. The hydrogen bonds are not directly involved in the chemical processes but are at some distance from the seat of reaction. The free energy profiles for the formation of enzyme-bound tyrosyl adenylate and the equilibria between the substrates and products were determined from a combination of pre-steady-state kinetics and equilibrium binding methods. By comparison of the profile of each mutant with wild-type enzyme, a picture is built up of how the course of reaction is affected by the influence of each side chain on the energies of the complexes of the enzyme with substrates, transition states, and intermediates (tyrosyl adenylate). As the activation reaction proceeds, the apparent binding energies of certain side chains with the tyrosine and nucleotide moieties increase, being weakest in the enzyme–substrate complex, stronger in the transition state, and strongest in the enzyme–intermediate complex. Most marked is the interaction of Cys-35 with the 3′-hydroxyl of the ribose. Removal of the side chain of Cys-35 leads to no change in the dissociation constant of ATP but causes a 10-fold lowering of the catalytic rate constant. It contributes no net apparent binding energy in the E·Tyr·ATP complex and stabilizes the transition state by 1.2 kcal/mol and the E·Tyr–AMP complex by 1.6 kcal/mol. The preferential stabilization of products causes the unfavorable equilibrium constant for the formation of Tyr–AMP and PP_i from Tyr and ATP in solution (3.5×10^{-7}) to be displaced to a value of 2.3 for enzyme-bound reagents. These experiments thus show that (i) the binding energies of side chains remote from the seat of reaction can be used to increase catalytic rates and (ii) the structure of regions of the binding site of an enzyme can be closer in complementarity to an unstable enzyme-bound intermediate than to the transition state for its formation.

The distinctive characteristic of enzyme catalysis compared with solution catalysis is that the enzyme specifically binds its substrate and can use the binding energy to enhance catalytic rate. Pauling (1946) suggested that an enzyme should be complementary in structure to the transition state of the substrate rather than to the substrate itself so that the enzyme would tend to deform the substrate into the transition state. Current ideas on the utilization of binding energy in catalysis support the concept of enzyme–transition-state complementarity but do not demand that the substrate is distorted by the enzyme. The presence of binding sites on the enzyme that form better bonds with the transition state of the substrate than with the unreacted substrate is sufficient to increase the turnover number of the enzyme, k_{cat} (Fersht, 1974, 1985; Jencks, 1975). Evidence has been adduced for the differential binding of transition state and substrate ("transition-state stabilization") from experiments in which the structure of substrates or inhibitors is varied [see Fersht (1985) for review]. But it is now possible to examine directly the catalytic role of binding energy of groups on an enzyme by performing experiments in which the structure of the enzyme is varied by site-directed mutagenesis (Winter et al., 1982; Fersht et al., 1984).

The tyrosyl-tRNA synthetase is particularly suited for such an analysis. Apart from favorable properties in handling and producing accurate kinetic data (Wilkinson, 1983), it is known from the direct solution of the crystal structure of the enzyme-bound tyrosyl adenylate complex combined with mu-

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