Activation of *Sulfolobus solfataricus* Alcohol Dehydrogenase by Modification of Cysteine Residue 38 with Iodoacetic Acid†

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ABSTRACT: Reaction of thermostable NAD^+-dependent alcohol dehydrogenase from *Sulfolobus solfataricus* with iodoacetate at pH 9.0 and 37 °C significantly increases the oxidation rate of aliphatic and aromatic alcohols and decreases the reduction rate of aromatic aldehydes. The archaeal ADH is chemically modified and activated in a Michaelis−Menten-type reaction, where one molecule of the reagent binds per active site. NAD^+ in micromolar concentration protects the enzyme against the inhibitor in an uncompetitive manner, while imidazole significantly increases the extent of the activation. Carboxymethylation selectively modifies one out of five cysteine residues per subunit, namely, Cys 38, located in the catalytic site, as determined by peptide and sequence analysis, and enhances by up to 25-fold the oxidation rate of benzyl alcohol. Carboxymethylated *Ss*ADH is less thermostable and shows a temperature optimum 30 °C lower than that of the native enzyme. The carboxymethylated enzyme exhibits a lower affinity toward the oxidized and reduced coenzyme. The dissociation constants for NAD^+ and NADH determined at 25 °C and pH 8.8 are 60- and 200-fold higher, respectively, compared to the native enzyme. The significant isotope effect in alcohol oxidation suggests that hydride transfer partially limits the turnover rate of the reaction catalyzed by the modified enzyme, whereas the rate-limiting step for the native enzyme is NADH dissociation. Carboxymethylated enzyme probably gives higher maximum velocities of oxidation because of the faster dissociation of the modified enzyme−coenzyme complex.

The alcohol dehydrogenase from the archaeon *Sulfolobus solfataricus* (*Ss*ADH)[1] is a thermostable NAD^+-dependent enzyme, structurally related to HLADH and YADH (Ammendola et al., 1992). Although the degree of sequence identity with respect to mesophilic ADHs is low (24−25%), most of the structurally and functionally important residues and adjacent regions are conserved or conservatively substituted. The archaeal ADH contains two zinc atoms per subunit. The catalytic zinc is bound to Cys 38, His 68, Cys 154, and a water molecule, while the structural zinc interacts with Glu 98 and three Cys residues at positions 101, 104, and 112 (Ammendola et al., 1992). All of these residues are located within sequence motifs typical of all zinc-containing ADHs (Vallee & Auld, 1990). The number of cysteine residues in the *Ss*ADH subunit is limited to five (all involved in the zinc binding) and is significantly lower than in YADH and HLADH, which have 8 and 14 cysteine residues per subunit, respectively.

A variety of cysteine-alkylating reagents have been used to investigate the structure and catalysis mechanism of HLADH. Cys 46 and Cys 174, both zinc ligands in the active site in HLADH, are selectively alkylated by iodoacetate and 3-bromopropionate, respectively, with almost complete loss of activity (Harris, 1964; Li & Vallee, 1963; Chadha & Plapp, 1984). The mechanism of inactivation of HLADH by halo acids has been established by kinetic studies (Reynolds & McKinley-McKee, 1969; Dahl & McKinley-McKee, 1981a; Chadha & Plapp, 1984) and indicates that a high-affinity, reversible enzyme inhibitor Michaelis−Menten complex is formed prior to covalent modification. The effects of carboxymethylation on the catalysis mechanism have been studied by steady-state and transient kinetics and by fluorescence binding (Reynolds & McKinley-McKee, 1975; Hardmann, 1976; Dahl & Dunn, 1984).

The X-ray structure of carboxymethylated HLADH has been determined to correlate the active-site region and the anion-binding site with the enzyme properties in solution (Zeppezauer et al., 1975) and, subsequently, to investigate the protein−coenzyme interactions (Cedergren-Zeppezauer et al., 1985). Selective carboxymethylation of Cys 174 by iodoacetate has been related to Arg and His at position 47 in the anion-binding site of $\beta_1\beta_1$ and $\beta_2\beta_2$ human liver isoenzymes, respectively (Bosron et al., 1986). More recently, differences in inactivation, labeling patterns, and the effect of buffers on alkylation have been reported for human $\gamma_1\gamma_1$ and $\beta_1\beta_1$ and equine EE ADH isoenzymes (Johansson et al., 1991).

Although chemical modification at the catalytic site in general leads to partial or complete loss of activity, an increase in activity was achieved in HLADH after picolinimidylation (Plapp, 1970) and in bovine lens aldose reductase after carboxymethylation (Liu et al., 1992). In contrast, we found that the treatment of coenzyme-free *Ss*ADH with iodoacetate produced an enhancement of its activity in benzyl

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[1] Abbreviations: ADH, alcohol dehydrogenase; HLADH, horse liver ADH; YADH, yeast ADH; *Ss*ADH, *Sulfolobus solfataricus* ADH; IAA, sodium iodoacetate; PE, *S*-pyridylethyl; BrImPpOH, DL-α-bromo-β-(5-imidazolyl)propionic acid; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid.

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alcohol oxidation and a change in its thermophilicity and thermostability. A preliminary account of this investigation has been reported (Raia et al., 1993). The present work describes the affinity labeling of *Ss*ADH with iodoacetate and compares the properties of the modified and the native enzyme.

MATERIALS AND METHODS

Protein. Alcohol dehydrogenase was prepared from *Sulfolobus solfataricus* DMS1617 strain, as described in Ammendola et al. (1992), with some modifications. The enzyme was obtained in a homogeneous form with a Blue A (Amicon) column with a specific activity of 2−4 units/mg. NAD^+ was removed by dialysis at 4 °C against 10−12 changes of 200 vol of 10 mM Tris-HCl (pH 8.1). The enzyme was then concentrated to 1−2 mg/mL by ultrafiltration on a PM30 Amicon membrane or under nitrogen flow and stored at 4 °C. Protein concentration was routinely determined with a Bio-Rad protein assay kit, using bovine serum albumin as standard. As judged by quantitative amino acid analysis, the concentration values obtained from the Bio-Rad assay were overestimated by 30%, so that they had to be normalized to effective values. The active-site concentration of enzyme was determined by titration with NAD^+ in the presence of excess pyrazole (Theorell & Yonetani, 1963) at 298 nm, pH 8.8, and 25 °C on the basis of a dimer of 75 kDa (Ammendola et al., 1992). Protein purity was checked by SDS−PAGE (Laemmli, 1970), denaturing the *Ss*ADH samples for 15 min at 100−120 °C in the presence of 2% SDS. Nondenaturing PAGE was carried out according to the Laemmli method with some modifications. SDS was omitted, and the pH of the electrode buffer was raised from 8.3 to 9.3.

Reagents. Sodium iodoacetate (IAA), iodoacetamide, DL-α-bromo-*β*-(5-imidazolyl)propionic acid (BrImPpOH), ADP, ADP−ribose, and CNBr were obtained from Sigma Chemical Co. (St. Louis, MO). Iodo[2-^{14}C]acetic acid was obtained from Radiochemical Centre (Amersham, U.K.). Bromoacetic acid, 3-iodopropionic acid, and 3-bromopropionic acid were from Aldrich and recrystallized from 70−80 °C petroleum ether before use. 4-Vinylpyridine was obtained from Sigma and used after vacuum distillation. Halo acid solutions were prepared in purified water (Milli-Q, Millipore) and titrated to about pH 5 with dilute NaOH. IAA dissolved in water gives a pH near 5, so that titration was unnecessary; solutions of 20−50 mM IAA were stable for several months at −20 °C. NAD^+ and NADH were purchased from either Fluka or Boehringer-Mannheim (grade I). Ethanol-d_6, 2-propanol-d_8, cyclohexanol-d_{12}, benzyl-d_7 alcohol, and pyrazole were obtained from Aldrich. Other chemicals used were of analytical grade.

Enzymatic Assay. The activity was assayed toward benzyl alcohol as described in Ammendola et al. (1992) with some modifications. Native enzyme was assayed at 65 °C in 1 mL of standard assay mixture containing 5 mM benzyl alcohol, 2 mM NAD^+, and 25 mM glycine/NaOH (pH 10.5). Carboxymethylated enzyme was assayed at 55 °C in 1 mL of assay mixture containing 20 mM benzyl alcohol, 5 mM NAD^+, and 25 mM glycine/NaOH (pH 10.5) (modified mixture). The pH of standard assay mixtures, at the respective assay temperatures, corresponded to the apparent optimal pH for native (8.5−9.1) and modified enzymes (9.2). Aliquots of enzyme solution (5−20 μL) were added to preheated cuvettes, and the increase or decrease in absorbance at 340 nm was recorded by using a Varian DMS200 spectrophotometer equipped with a thermostated water bath or a Cary 1E spectrophotometer equipped with a computer-controlled temperature system. Enzyme units were expressed as micromoles of NADH produced per minute on the basis of an absorbance of NADH of 6.22 mM^{-1} cm^{-1} at 340 nm. Kinetic studies on alcohols and aldehydes were performed by using the preceding glycine buffers or 25 mM Tris-HCl (pH 7.5), respectively. For the comparison of kinetic parameters, the catalytic activity of the modified and unmodified enzymes was assayed at the same temperature of 55 °C.

Each series of kinetic parameter measurements was carried out in duplicate. Kinetic results were analyzed by using the Enzfitter program (Elsevier-Biosoft, Cambridge) to calculate K_m and V_{max}. Coefficients of variation were around 5% for V_{max} and 16% for K_m estimates. Solutions of NADH were prepared daily in Tris buffer, while stock solutions of NAD^+ were prepared in water and stored frozen at −20 °C. The stability of the coenzyme solutions was checked by determining the absorption quotients A_{250}/A_{260} and A_{280}/A_{260} for NAD^+ and A_{260}/A_{340} for NADH, as indicated by the manufacturer.

*Reaction with Alkylating Reagents***.** Samples of *Ss*ADH at 1−2 mg/mL protein concentration in 50−100 mM Tris-HCl (pH 9.0) were preincubated at 37 °C under subdued light, and 5−10 μL aliquots were withdrawn and assayed at 50 °C in the modified mixture. Alkylating reagent solution (2−5 μL) was then added to the enzyme (molar ratio = 100:1 over the dimer), and the activity was assayed in aliquots withdrawn at intervals of time. The possibility of further alkylation during the activity assay was excluded, as the presence of 2-mercaptoethanol in the assay mixture did not affect the rate of enzyme reaction. However, glycine present in the assay mixture acted as a scavenger for iodoacetate (Syversten & McKinley-McKee, 1981).

Preparation of Carboxymethylated SsADH. Modified enzyme was prepared by incubating 2−3 mg of native enzyme with IAA under the conditions described earlier and following the enhancement of the enzymatic activity until it reached a maximum. The reaction mixture was immediately treated with 2-mercaptoethanol in 10-fold molar excess over IAA and loaded onto a Sephadex G-25 Fine column equilibrated with 0.1 M Tris-HCl (pH 8.0). The active pool from gel filtration was concentrated under nitrogen flow, and the specific activity of the modified enzyme was determined. Carboxymethylated *Ss*ADH, at 1−2 mg/mL, was stored frozen in 20% glycerol for up to 2 weeks without an appreciable loss of activity.

Alkylation with Iodo[2-^{14}C]acetate. Purified alcohol dehydrogenase, extensively dialyzed to remove the coenzyme, was treated in the absence and presence of NAD^+. In the first case, the enzyme (13 nmol in 0.5 mL of 0.1 M Tris-HCl, pH 9.0) was treated with a 90-fold molar excess of iodo[2-^{14}C]acetate (20 μCi/μmol) at 37 °C until the enzymatic activity reached a maximum. The labeled enzyme (specific activity = 21.000 cpm/nmol) was immediately treated with 2-mercaptoethanol (10-fold molar excess over iodoacetate) and purified by gel filtration as described earlier. The active pool was concentrated under nitrogen flow, and the incorporation of [^{14}C]carboxymethyl groups per mole of

ADH was determined by liquid scintillation using Dynagel (Baker, Holland) and a Packard Tri-Carb 300 β-counter ([^{14}C]-sample A).

In a parallel experiment, the enzyme (12 nmol) was treated with iodo[2-^{14}C]acetate in the presence of 1 mM NAD^+ using the same experimental conditions. The reaction was stopped at the same time as in the previous experiment by adding 2-mercaptoethanol, followed by gel filtration. The active pool, containing only a background level of radioactivity, was concentrated as previously reported (sample B).

Alkylation with 4-Vinylpyridine. The [^{14}C]carboxmethylated enzyme (10 nmol of [^{14}C]sample A) and the same amount of sample B were reduced with a 30:1 molar excess of DTT over SH groups in 0.2 M Tris-HCl (pH 8.0) containing 2 mM EDTA and 6 M guanidine hydrochloride for 3 h at 30 °C under a nitrogen atmosphere; 4-vinylpyridine (10:1 molar excess over DTT) was added to each sample, and the reaction was allowed to proceed in the dark for 2 h at 25 °C under a nitrogen atmosphere. The excess reagent and bioproducts were removed by dialysis against 9% formic acid.

Cleavage with CNBr and Separation of Fragments. Both samples (PE−[^{14}C]sample A and PE−sample B) were withdrawn under nitrogen flow and redissolved in 70% formic acid; CNBr (dissolved in acetonitrile) was added to each sample (80-fold molar excess over methionine), and the reaction was allowed to proceed in the dark at room temperature under a nitrogen atmosphere for 24 h (Fontana & Gross, 1986). The resulting peptide mixtures were freeze-dried in a Savant concentrator and separated by RP-HPLC on a μBondpak C_{18} column (0.39 × 30 cm) from Waters-Millipore (Milford, MA). Eluent A was aqueous 0.1% trifluoroacetic acid and eluent B was 0.07% trifluoroacetic acid in acetonitrile. Elution was accomplished at a flow rate of 1 mL/min and was monitored by the absorbance at 220 nm. Peaks were collected manually and freeze-dried. The presence of a [^{14}C]carboxymethyl)cysteine-containing peptide in the PE−[^{14}C]sample A digest was determined by liquid scintillation counting.

Sequence Analyses. The purified ^{14}C-labeled CNBr peptide of the PE−[^{14}C]sample A and the corresponding CNBr peptide of PE−sample B were dissolved in aqueous 0.1% trifluoroacetic acid (30−60 μL) and submitted to automated sequence analysis by using a pulsed, liquid-phase automatic sequencer (Applied Biosystems Model 477A) equipped with an on-line phenylthiohydantoin amino acid derivative analyzer (Model 120A). Samples were loaded onto a trifluoroacetic acid-treated glass fiber filter coated with polybrene and washed according to the manufacturer's instructions. The relative reagents were from Applied Biosystems.

Amino Acid Analyses. Amino acid analyses of PE−[^{14}C]-sample A and PE−sample B were performed after hydrolysis at 110 °C *in vacuo* for 24, 48, and 72 h in 6 M HCl and analyzed with a Carlo Erba Model A329 amino acid analyzer. Cysteine content was evaluated both as (carboxymethyl)-cysteine and as (pyridylethyl)cysteine.

Effect of pH. The effect of pH on benzyl alcohol oxidation and benzaldehyde reduction for both native and carboxymethylated enzymes was determined at 55 °C, between pH 7.0 and 11.0, in the respective standard assay conditions, except that different buffer systems were used.

Thermophilicity Studies. Native and carboxymethylated enzymes were assayed in the temperature range of 30−90 °C, according to the standard assay conditions, using 3−6 μg of protein/mL of assay. Studies on heat stability were carried out by incubating 0.5 mg/mL protein in 50 mM Tris-HCl (pH 8.5) in stoppered plastic tubes at different temperatures. At intervals of time, the samples were centrifuged and small aliquots were withdrawn and assayed.

Circular Dichroism and Fluorescence Studies. CD spectra were recorded with a JASCO J-710 spectropolarimeter equipped with computer-controlled temperature cuvette holders. Thermal analysis was performed between 25 and 90 °C on 0.2 mg/mL protein samples in 10 mM Tris-HCl (pH 8.0) using 1.0 mm path length cells. The temperature of the sample cell was increased in 10 °C increments, and when the desired temperature was obtained the sample was allowed to equilibrate for 3 min. The spectra were then registered between 240 and 200 nm and represent the average of at least five tracings.

The binding of NAD^+ and NADH to native and modified *Ss*ADH was investigated by fluorescence quenching measurements carried out at 25 °C in the presence of 25 mM Tris-HCl (pH 8.8), using a JASCO FP-777 spectrofluorometer equipped with an external thermostated water bath. Aliquots (5 μL) of 2-200 μM NADH or 25-1000 μM NAD^+ in Tris buffer (pH 8.8) were added to 500 μL of enzyme solution (0.2 μM dimer) excited at 280 nm in a quartz fluorescence cuvette, and the fluorescence emission was monitored at 322 and 326 nm for native and modified *Ss*ADH, respectively.

The treatment of binding data to determine the dissociation constant, K_d, and the number of binding sites, n, was performed by three different methods, using the Enzfitter program. ΔF_{max} (maximal fluorescence change with complete saturation) was evaluated from the linear part of the double-reciprocal plot ($1/\Delta F$ versus $1/L$, where L is the concentration of coenzyme added); the molar fraction (α) was obtained at each coenzyme concentration from the ratio $\Delta F/\Delta F_{max}$, on the assumption that the fluorescence change is directly proportional to the amount of enzyme−coenzyme complex. The quantities αE_0 (where E_0 is the initial enzyme normality) and $L - \alpha E_0$ give the concentrations of bound (L_b) and free (L_f) coenzyme in solution, respectively, after which the Scatchard equation was applied (Iweibo & Weiner, 1972). The Hill equaton was also applied: $(\ln(\alpha/1 - \alpha) = h \ln L_f - \ln K_d$, where h, the Hill coefficient, and K_d are constants determinable from the slope and intercept of the curve, respectively (Luisi et al., 1973). K_d and n were also determined by plotting L/α versus $l/(1 - \alpha)$, according to Bagshaw and Harris (1987). Solutions were diluted and the inner filter effect was neglected (Bagshaw & Harris, 1987). The titrations were performed in duplicate or triplicate. Coefficients of variation were less than 5% for K_d and n estimates.

Studies with Deuterated Alcohols. The catalytic activity of the unmodified and modified *Ss*ADH on ethanol, 2-propanol, cyclohexanol, and benzyl alcohol was measured at 55 °C with saturating concentrations of alcohol and NAD^+, and the values of V_{max} were compared to those obtained with the corresponding deuterated alcohols under the same experimental conditions. Each measurement was carried out in duplicate.

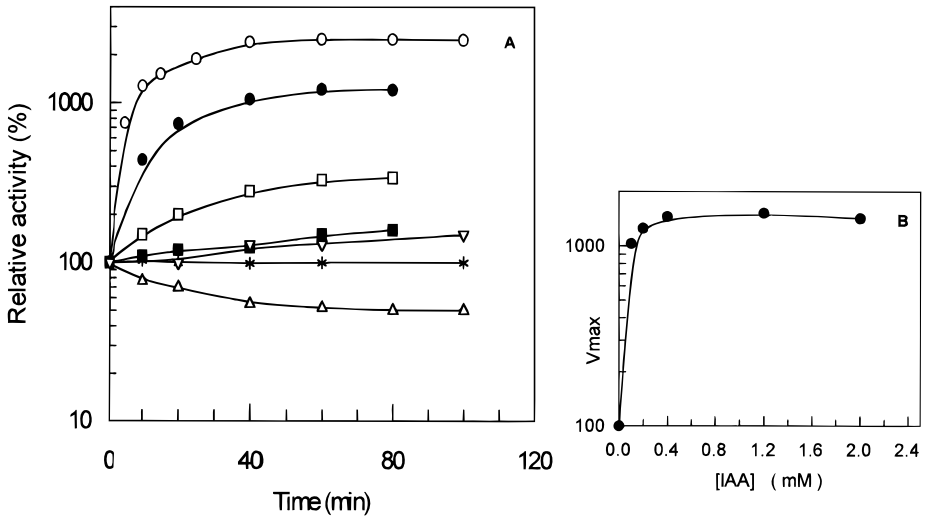

FIGURE 1: (A) Effect of halo acids on the enzyme activity of *Ss*ADH. Enzyme (12 μM) was incubated at 37 °C in the presence of 1.0 mM IAA (○, △), bromoacetate (●), 3-iodopropionate (□), 3-bromopropionate (■), and 1.0 mM IAA plus 30 μM NAD^+ (▽). The activity was measured at 50 °C on aliquots withdrawn at intervals of time, using benzyl alcohol as substrate (except for △, where benzaldehyde was used). The activity as a function of the time of incubation is presented on a semilog plot, as percent relative to the control (*) without IAA at zero time. (B) Dependence of *Ss*ADH activation on IAA concentration. Values of v_{max} represent the maximal activity obtained with different IAA concentrations and are given relative to a control without IAA (abscissa).

RESULTS

Effect of Alkylating Reagents. Figure 1A shows the effect of halo acids on the *Ss*ADH activity. When benzyl alcohol was used as substrate, higher activation was obtained by IAA, while a slight activating effect was observed with 3-bromopropionate. When benzaldehyde was used as substrate, the activity decreased to about 50% with respect to the initial value upon alkylation by IAA (Figure 1A). Under the specified conditions, the activity toward benzyl alcohol increased by up to 25-fold in 60 min with respect to the initial value, depending on the specific activity of the purified enzyme and the presence of coenzyme not completely removed by dialysis. The activity of the enzyme modified with IAA decreased very slowly after reaching the maximum value. After 20 h of reaction with IAA at 37 °C, the activity was 80% with respect to the maximum value. The same decrease in activity was observed when 2-mercaptoethanol was added after 80 min to quench the reaction of IAA while keeping the sample at 37 °C.

Other common SH-alkylating reagents of proteins, such as *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and iodoacetamide, partially inactivated the enzyme (data not shown). The activity of the enzyme was not affected when BrImPpOH was used at pH 8.1 as the alkylating agent during 60 min of reaction; after this time, the addition of iodoacetate to the mixture produced a significant increase in dehydrogenase activity.

Activation by Iodoacetic Acid. The enhancement of the dehydrogenase activity by reaction with different concentrations of IAA is shown in Figure 1B. Increasing concentrations of the reagent did not cause a further increase in the catalytic activity, but produced a slight decrease, similar to that observed after a long reaction in the presence of lower IAA concentrations. The rate of activation of *Ss*ADH by IAA was examined as a function of time, and the velocity data were fit to the following expression for the pseudo-first order increase in activity:

$$v_t = v_0 + v_{max}(1 - e^{-k_{obs}t})$$

where v_0 is the initial velocity of *Ss*ADH, v_{max} is the stationary value of v_t that is approached asymptotically as $t \rightarrow \infty$, and k_{obs} is the pseudo-first-order rate constant for the activation. A semilog plot of $v_{max} - v_t$ versus time was linear, indicating that the kinetics of activation was pseudo-first-order with respect to enzyme in the presence of excess of IAA (Figure 2A). The value of k_{obs}, obtained by the slope of the semilog plot, was found to be dependent on the IAA concentration in a hyperbolic manner. This behavior is indicative of an active-site-directed mechanism, as described in the equation

$$E + A \overset{K_A}{\rightleftharpoons} E{\cdot}A \xrightarrow{k_2} EA$$

where rapid reversible binding of the affinity label (A) to the enzyme (E) precedes the covalent modification to an (in)-active complex EA (Syversten & McKinley-McKee, 1981; Plapp, 1982). Figure 2B shows the double-reciprocal plot of k_{obs} against the IAA concentration, which fits the equation $k_{obs} = k_2[A]/(K_A + [A])$ and allows the determination of the maximal rate constant for the activation, k_2, and the dissociation constant, K_A. The values of k_2 and K_A were 0.14 $\pm$ 0.01 min^{-1} and 1.0 $\pm$ 0.2 mM, respectively; the pseudobimolecular rate constant for the activation (k_2/K_A) was 2.3 M^{-1} s^{-1}. The value of K_A is similar to that determined for the carboxymethylation of HLADH, although under different reaction conditions (Dahl & McKinley-McKee, 1981a). The overall rate of activation is about 4 times less than the constant for the reaction of IAA with the thiolate anion of cysteine and 180 times larger than the constant for the reaction of IAA with the mercaptoethanol−zinc complex (Dahl & McKinley-McKee, 1981a).

The preceding results indicate that IAA activates *Ss*ADH by selective carboxymethylation of one or more SH groups in the catalytic site through an active-site-directed mechanism and that the affinity labeling is facilitated by the binding of the reagent to the enzyme. To further evaluate the interaction of IAA with *Ss*ADH, the activation was analyzed in the presence of NAD^+. Figure 1A shows that *Ss*ADH is poorly

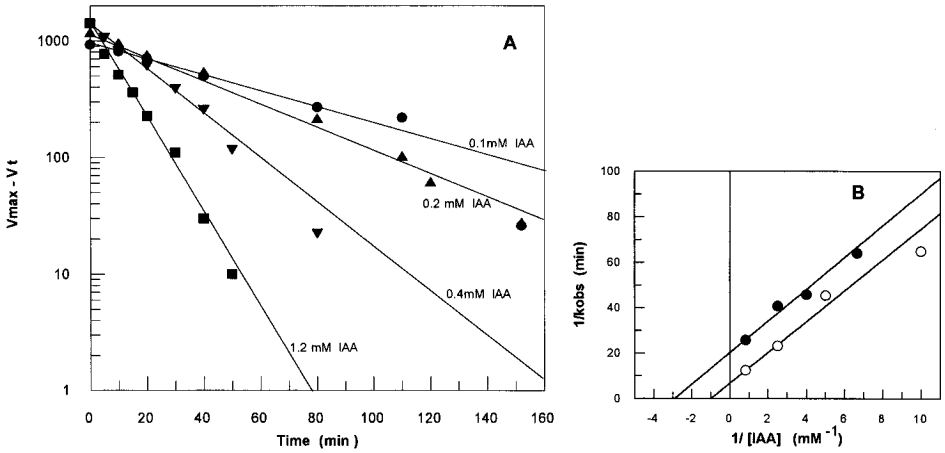

FIGURE 2: (A) Semilog plot derived from the equation, $v_t = v_0 + v_{max}(1 - e^{-kobst})$, where v_{max} is the maximal activity to which v_t approximates at large time. Enzyme (10 μM) was incubated with the indicated amount of IAA and assayed with benzyl alcohol, as described in Figure 1. Slope $= -0.43k_{obs}$. (B) Double-reciprocal plot of rate constants versus IAA concentrations, determined in the absence of NAD^+ (○, data from part A) and the presence of 11 μM NAD^+ (●). Enzyme concentration was 9 μM. Experimental conditions same as for Figure 1. Constant values were determined using the *GraFit* program (Leatherbarrow, 1992).

activated in the presence of a slight excess of NAD^+, precluding the determination of the activation parameters at higher coenzyme concentrations. A 10-fold molar excess of NAD^+ over the dimer, i.e., 5 times over the binding sites, fully protected the enzyme (data not shown). However, in the presence of less than stoichiometric NAD^+ concentrations and different IAA concentrations, the k_2 and K_A values that resulted were 0.05 ± 0.04 min^{-1} and 0.35 ± 0.07 mM, respectively, indicating that the affinity of IAA for the protein is nearly unchanged and that the protection by the coenzyme is uncompetitive (Figure 2B); under these conditions the v_{max} that resulted was about 50% of that measured in the absence of NAD^+. The values of k_{obs} and the related reagent concentrations (from Figure 2B) were also used to determine the order of the activation reaction, *n*, with respect to the reagent, according to Levy et al. (1963); this is given by the slope of the log k_{obs} versus log[IAA] plot and gives an indication of the stoichiometry of activation. The values of *n* that resulted were 0.80 ± 0.05 and 0.45 ± 0.04 for the alkylation reaction in the absence and presence of NAD^+, respectively (data not shown). This indicates that one molecule of IAA is able to activate one active site.

ADP and ADP−ribose, added to the enzyme in a 6-fold molar excess over dimer, protected the enzyme against activation by IAA to a lesser extent than did NAD^+. In fact, after 60 min of incubation with IAA, the activity enhancement was 70% with ADP and 50% with ADP−ribose, with respect to a control without ligand.

Effect of Buffers. Activation curves with 10 μM *Ss*ADH and 1.0 mM IAA, at 37 °C, in the presence of different buffers were obtained as described earlier, and the pseudo-first-order constants were calculated from the semilog plot of $v_{max} - v_t$ against *t*. The k_{obs} and v_{max} values for five different reaction conditions are listed in Table 1. The presence of phosphate ions or high chloride concentrations appears to decrease the alkylation reaction, while imidazole gives higher activation.

Identification of the Carboxymethylated Cysteine. The *Ss*ADH treated with iodo[2-^{14}C]acetate in the absence of NAD^+ was found to be radioactive, with about 1 mol of [^{14}C]carboxymethyl groups/mol of monomer (0.9 ± 0.1, as determined by an average of three independent determinations); on the contrary, no radioactivity was found when alkylation with [^{14}C]IAA was performed in the presence of 1 mM NAD^+. After extensive alkylation of both samples with 4-vinylpyridine, amino acid analysis revealed the presence of five and four (pyridylethyl)cysteine residues in the unlabeled (PE−sample B) and labeled (PE−[^{14}C]sample A) proteins, respectively; moreover, only one (carboxymethyl)cysteine was identified in the [^{14}C]carboxymethylated enzyme.

Table 1: Effect of Buffers on the Alkylation Reaction of IAA with *Ss*ADH[a]

buffers	k_{obs} (min^{-1})	v_{max} (%)
75 mM Tris-HCl, pH 9.1	0.12	100
10 mM Tris-HCl, pH 8.1	0.14	80
10 mM Tris-HCl, pH 8.1 + 0.1 M NaCl	0.02	75
35 mM sodium phosphate in 5 mM Tris-HCl, pH 8.1	0.06	90
17 mM imidazole in 5 mM Tris-HCl, pH 8.1	0.13	130

[a] The enzyme (10 μM) was treated with 1.0 mM IAA in the indicated buffers at 37 °C. The maximum activity and the pseudo-first-order rate constant were determined as described in the Materials and Methods and Results sections, respectively.

After digestion of both samples with CNBr, the resulting peptide mixtures were fractionated on RP-HPLC. Figure 3 shows the separation of fragments relative to the PE−[^{14}C]-carboxymethylated enzyme. The elution profile was similar to that already reported (Ammendola et al., 1992); only one peak (CB1) was found to be radioactive, whereas only background radioactivity was found throughout the column. The yield of CB1 peptide was about 20%, due to its length (44 amino acids) and hydrophobic nature. Sequencing of this peptide (1 nmol) allowed the identification of the unique [^{14}C]carboxymethylated Cys at position 38 of the sequence; in addition, radioactivity (800 cpm) was found only in this cycle of the Edman degradation. An identical sequence was determined for the corresponding fragment obtained after digestion of the enzyme treated with iodo[2-^{14}C]acetate in the presence of NAD^+, except for the presence of a (pyridylethyl)cysteine derivative in the same position. So, by analogy to HLADH, IAA selectively alkylates one out of two SH ligands of the *Ss*ADH catalytic zinc.

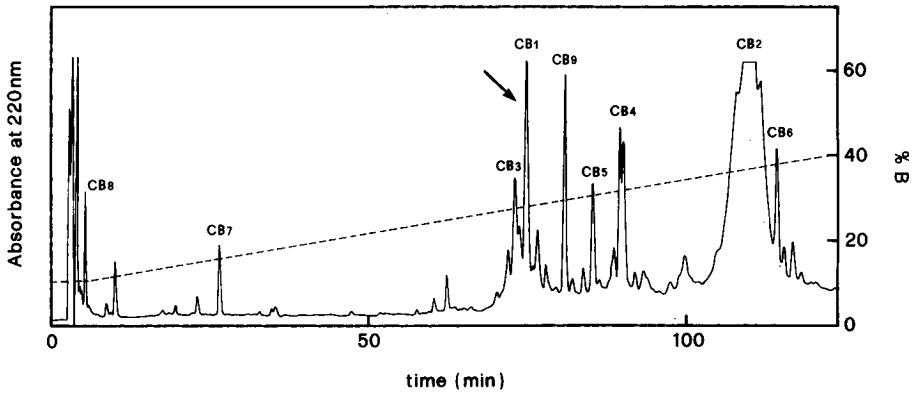

FIGURE 3: HPLC profile of peptides generated by CNBr digestion of PE−[^{14}C]carboxymethylated *Ss*ADH. The column was a C_{18} μBondapak. Solvent A was 0.1% TFA; solvent B was 0.08% TFA in acetonitrile. Flow rate = 1 mL/min. Absorbance was at 220 nm. The arrow indicates the only peak containing radioactivity.

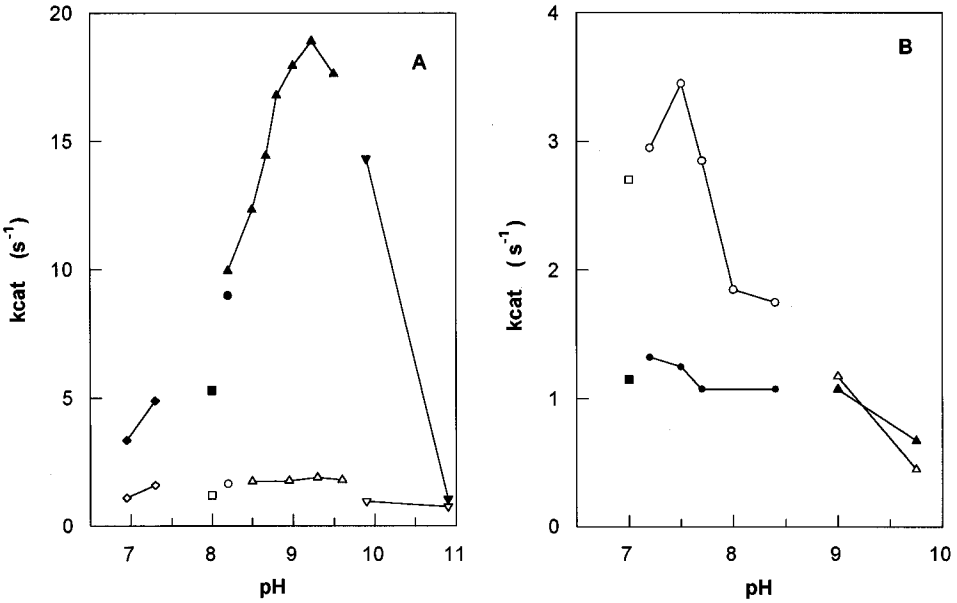

FIGURE 4: pH dependence of the activity of native (open symbols) and modified *Ss*ADHs (filled symbols) on benzyl alcohol (A) and benzaldehyde (B), as measured at 55 °C. The following buffer systems were used. In (A): (◇, ◆) 25 mM sodium phosphate; (□, ■) 50 mM Taps/HCl; (○, ●) 50 mM Tris/H_3PO_4; (△, ▲) 25 mM glycine/NaOH; (▽, ▼) 24 mM disodium hydrogen phosphate/NaOH. In (B): (□, ■) 25 mM sodium phosphate; (○, ●) 25 mM Tris/HCl; (△, ▲) 25 mM glycine/NaOH. pH values were controlled in the assay mixture at 55 °C.

Characterization of Carboxymethylated SsADH. The carboxymethylated enzyme, prepared as described in the Materials and Methods section, migrated slightly more than the native enzyme, essentially as a single band in nondenaturing PAGE (data not shown). The specific activity of the modified enzyme was 5−10-fold higher than that of the native enzyme, as determined by the respective standard assay conditions. To study the kinetic behavior of the modified and unmodified enzymes at their optimal pH, the pH dependencies of the reduction and oxidation reaction rates were compared (Figure 4). Apparent optimal pH was approximately the same for both enzymes, but the dehydrogenase activity of the modified enzyme changed sharply as a function of pH.

The effect of temperature on dehydrogenase activity is shown in Figure 5. The reaction rate of the native enzyme increased up to an instrumental limit of 90 °C, while that of the modified enzyme increased more markedly up to about 65 °C and then decreased abruptly; the corresponding Arrhenius plots are shown in the inset of Figure 5. The energy of activation values were 11.8 ± 0.6 and 11 ± 1 kcal/mol for the native and modified enzymes, respectively (average of three determinations). The thermostability of native and modified *Ss*ADH was examined at two different temperatures. The native enzyme remained completely stable (i.e., no loss of activity) after heating for 3 h at 55 °C at 0.5 mg/mL protein concentration, while the modified enzyme lost 10% of activity under the same conditions. At 65 °C the half-lives of the native and modified enzymes were 9 and 4 h, respectively.

Because the carboxymethyl group introduced produced a significant effect on the stability of the *Ss*ADH, it was of interest to correlate alterations in protein conformation with thermostability. Far-UV CD measurements were performed as a function of temperature to monitor changes in the secondary structure of both enzymatic forms. At 25 °C, the CD spectrum of the carboxymethylated enzyme was similar to that of the native enzyme, with both showing the features typical of an α-helical protein, i.e., the presence of distinct peaks centered at about 208 and 222 nm (data not shown). On the other hand, no difference in the CD spectra could be detected for native *Ss*ADH from 25 to 90 °C, while the CD

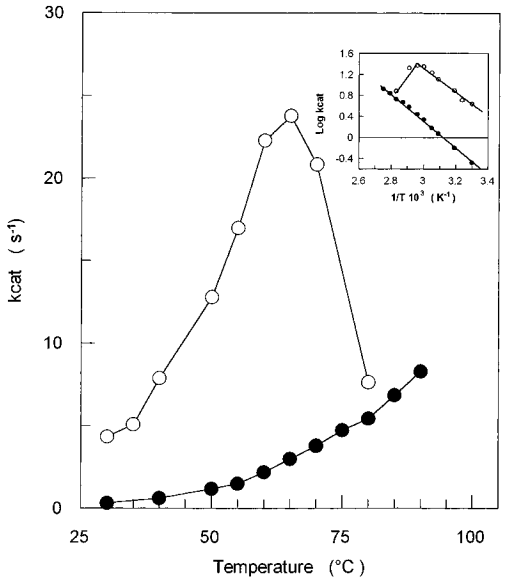

FIGURE 5: Dependence of the native (●) and modified (○) *Ss*ADH activities on the temperature. The assays were carried out as described in Materials and Methods. In both experiments, NAD^+ and benzyl alcohol concentrations were saturating at all temperatures. The inset shows the Arrhenius plot of the same data.

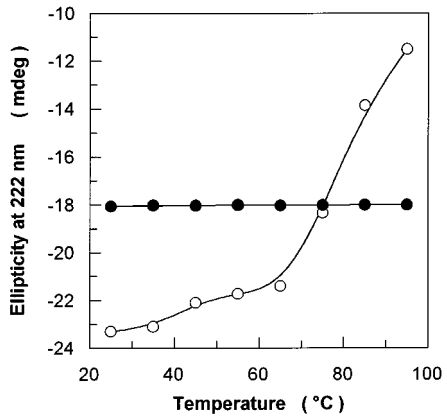

FIGURE 6: Temperature dependence of the dichroic activity at 222 nm for native (●) and modified (○) *Ss*ADHs. Experimental tracings were accumulated and corrected for blanks by computer. Each spectrum is the average of at least five tracings, registered at the indicated temperature, as described in Materials and Methods. Protein concentration was 1.9 μM as the dimer in 10 mM Tris-HCl (pH 8.1).

curves of modified *Ss*ADH significantly changed their shape and intensity. Figure 6 reports the dichroic activity at 222 nm as a function of the temperature for both enzymatic forms. An abrupt decrease in dichroic activity is evident around 65 °C for the modified enzyme, in accordance with the sharp decrease in enzyme activity observed at this temperature.

Coenzyme Binding Studies. The shape of fluorescence emission spectra of the native and carboxymethylated *Ss*ADHs appeared similar upon excitation at 280 nm, with the emission maxima centered at 322 and 326 nm, respectively, and the intensity of the modified enzyme quenched by 25% with respect to the native enzyme. The λ_{max} values indicated that both of the tryptophans present on the enzyme subunit, Trp 95 and Trp 117 (Ammendola et al., 1992), are shielded from the solvent, although the microenvironment surrounding one or both these residues seems more polar in the modified enzyme. Moreover, excitation of the native *Ss*ADH at 295 nm, pH 8.8, and 25 °C in the presence of excess of NADH resulted in the appearance of a second peak at about 422 nm in the emission spectrum, which was due to fluorescence energy transfer from the Trp residue(s) to the bound coenzyme with the aromatic residue evidently being within Förster distance (Stryer, 1978) of the coenzyme binding site (data not shown). Kinetics and fluorescence studies with chiral and achiral alcohols have suggested that the substrate specificity of *Ss*ADH is affected by Trp 95, located in the substrate-binding pocket, in the same way as Trp 93 and Phe 93 affect YADH and HLADH, respectively (Raia et al., 1994; Marino et al., 1995; Green et al., 1993). Because charged groups are well-known quenchers of nearby fluorophores (Brand & Witholt, 1967; Wiget & Luisi, 1978), we can hypothesize that the Trp 95 is quenched by the proximal charged carboxyl group bound to Cys 38. However, the possibility that an energy transfer to ionized tyrosine and/or a slight conformational rearrangement in the structure near Trp 95 are involved in the quenching process, bringing a quenching group into contact with the indole ring, is not excluded. Detailed fluorescence quenching studies would be necessary to analyze this matter.

Titration with NAD^+ and NADH of native *Ss*ADH produced relevant protein fluorescence quenching (about 60%). The binding data (plotted according to three methods of calculation) gave linear plots that produced binding constants that differed from each other by no more than 10%. The estimated mean values of K_d for NAD^+ and NADH binding to native *Ss*ADH at pH 8.8 and 25 °C were 0.50 and 0.05 μM, respectively, with two binding sites per dimer and Hill coefficients equal to unity. A similar binding plot for NAD^+ was obtained at 37 °C and pH 9.0, which produced a K_d value of 0.45 μM and two binding sites per dimer. The fluorescence of the modified *Ss*ADH required a higher coenzyme concentration to be quenched to the same extent that was observed for native enzyme under the same experimental conditions. Binding data for both NAD^+ and NADH fit linear Scatchard plots, although occasionally a slight upward curvature was found in the first part of the plot that included the 10−15% of the total experimental data of binding. This apparent biphasicity, although not very meaningful in a region where the Scatchard plot is highly sensitive, i.e., for $\alpha \leq 0.2$, could be due to either the heterogeneity of the sample or negative cooperativity between the two binding sites of the modified enzyme. The values of K_d and n calculated from extrapolation of the linear part of the curves were 29 μM and 2 for NAD^+ and 11 μM and 2 for NADH, respectively. Hill coefficients for both NAD^+ and NADH were equal to unity. Thus, it appears that NAD^+ and NADH bind more weakly to the modified *Ss*ADH than to native enzyme, with the respective K_d values being increased by about 60- and 200-fold.

Kinetic Characterization. A comparison of the steady-state kinetic parameters of the native and carboxymethylated enzymes, determined at 55 °C with various substrates, is reported in Table 2. Of all of the alcohols tested, cyclohexanol was the best substrate for the native enzyme: it has a specificity constant (k_{cat}/K_m) 20-fold greater than that of ethanol, followed by monobromobenzyl alcohols with a value 12-fold greater. More than the catalytic rate, it is the substrate affinity that contributes to the high k_{cat}/K_m value observed for cyclohexanol, which has a relatively low K_m. The reverse reaction has been studied using benzaldehyde

Table 2: Kinetic Constants for the Native and Carboxymethylated *Ss*ADHs[a]

substrate	*Ss*ADH k_{cat} (s^{-1})	*Ss*ADH K_m (mM)	*Ss*ADH k_{cat}/K_m (s^{-1} mM^{-1})	CM*Ss*ADH k_{cat} (s^{-1})	CM*Ss*ADH K_m (mM)	CM*Ss*ADH k_{cat}/K_m (s^{-1} mM^{-1})
ethanol	0.5	0.70	0.7	0.85	184	0.004
1-propanol	1.2	0.33	3.6	2.2	2.4	0.9
2-propanol	0.25	0.60	0.4	0.45	53	0.008
cyclohexanol	0.65	0.045	14.4	1.2	0.23	5.3
benzyl alcohol	1.4	0.80	1.7	14.5	2.0	7.2
3-methoxybenzyl alcohol	1.4	1.20	1.1	16.0	3.3	4.8
4-methoxybenzyl alcohol	1.5	1.30	1.1	20.5	1.4	14.6
3-bromobenzyl alcohol	1.2	0.14	8.6	2.0	0.9	2.2
4-bromobenzyl alcohol	1.1	0.12	9.1	3.5	0.4	8.7
NAD^+ (benzyl alcohol)	1.4	0.20	7.0	18.5	0.5	37.0
benzaldehyde	3.5	0.30	11.6	1.8	0.2	9.0
4-methoxybenzaldehyde	0.6	0.14	4.3	NA[b]		
4-carboxybenzaldehyde	0.7	0.28	2.5	NA[b]		
4-nitrobenzaldehyde	4.6[c]		2.4[c]			
NADH (benzaldehyde)	4.5	0.03	150	2.0	0.04	50.0

[a] The activity was measured at 55 °C as described in Materials and Methods using NAD^+ and NADH in saturating concentrations. Kinetic constants for NAD^+ and NADH were determined with saturating concentrations of benzyl alcohol and benzaldehyde, respectively. k_{cat} is the turnover number of the forward and the reverse reactions. [b] NA, no measurable activity. [c] From the apparent V_{max} measured in the presence of 2% acetonitrile added to solubilize the 4-nitrobenzaldehyde. This substrate was present at the concentration corresponding to that of benzaldehyde necessary to saturate the enzyme; 1−2% acetonitrile significantly inactivates the enzyme already, precluding the precise determination of kinetic data. CM*Ss*ADH, carboxymethylated *Ss*ADH.

and substituted benzaldehydes. Benzaldehyde is less reactive than 4-nitrobenzaldehyde and more reactive than 4-methoxy- and 4-carboxybenzaldehyde. In the latter case, the catalytic rate, rather than the affinity for substrate, affects the specificity constant. At fixed saturating concentrations of substrates, the native enzyme oxidizes NADH with a specificity constant 20-fold greater than that found for NAD^+ oxidation.

Carboxymethylation changes the catalytic pattern of *Ss*ADH considerably, affecting both its specificity and efficiency. The affinity of *Ss*ADH decreased for all the alcohols tested upon carboxymethylation, as indicated by K_m values that, for example, are increased by up to 250-fold for ethanol and 90-fold for 2-propanol. On the other hand, the catalytic rate doubled for aliphatic alcohols and increased by over 10-fold for benzyl and monomethoxybenzyl alcohols. Interestingly, the modified enzyme seems to have acquired the capacity to distinguish the *meta* from the *para* position in substituted benzyl alcohols, unlike the native enzyme. In fact, the k_{cat}/K_m values of 3-methoxy- and 3-bromobenzyl alcohols are identical or similar to those of the corresponding 4-derivatives for the native enzyme, while they are 3−4-fold lower for the modified enzyme. For the reverse reaction, the modified enzyme shows decreased activity toward benzaldehyde, as already observed in Figure 1, and only a poor activity toward 4-methoxy- and 4-carboxybenzaldehyde. The specificity constant for NAD^+ reduction is increased 5-fold, while that for NADH oxidation is decreased 3 times, upon enzyme modification.

Table 3: Primary Deuterium Kinetic Isotope Effects on the Native and Carboxymethylated *Ss*ADHs

substrate	$V_{H/D}$[a] native *Ss*ADH	$V_{H/D}$[a] CM*Ss*ADH
ethanol($-d_6$)	1.2	3.9
2-propanol($-d_8$)	1.3	2.8
cyclohexanol($-d_{12}$)	1.5	3.0
benzyl alcohol($-d_7$)	1.1	3.1

[a] $V_{H/D}$ represents the ratio between the V_{max} values for nondeuterated and deuterated alcohol oxidation, measured at 55 °C at saturating concentrations of both alcohol and coenzyme. CM*Ss*ADH, carboxymethylated *Ss*ADH. Standard errors were less than 15%.

Deuterium Kinetic Isotope Effect. Table 3 lists the kinetic isotope effects on V_{max}^H/V_{max}^D determined on native and carboxymethylated *Ss*ADHs. With deuterated ethanol, 2-propanol, cyclohexanol, and benzyl alcohol as substrates, a V_{max}^H/V_{max}^D ratio of about 1 was observed for the native enzyme, while for the modified enzymes there were deuterium isotope effects of 3.9, 2.8, 3.0, and 3.1, respectively.

DISCUSSION

The pattern of different reactivities previously observed for a series of halo acids in HLADH inactivation (Dahl & McKinley-McKee, 1981a) is also observed in the *Ss*ADH activation studied in the present work. In fact, the haloacetates are more reactive than β-halopropionates, and iodoacetate is about twice as reactive as bromoacetate, as expected from chemical reactivity. Moreover, this behavior agrees with the dissimilar chemical reactivities that halo acids have toward the free thiolate anion of cysteine in similar experimental conditions, i.e., pH 9.0 and 37 °C, and also toward zinc−thiol complex, as reported by Dahl and McKinley-McKee (1981a,b). The decrease in activity at high concentrations of IAA could be due to the alkylation of other cysteine residues presumedly less reactive than those involved in the activation process. This hypothesis is suggested by the alkylation of three cysteine ligands of the structural zinc atom in addition to Cys 46 in HLADH, upon treatment with IAA in high excess (Cedergren-Zeppezauer et al., 1985). However, the possibility that the excess reagent reacted with other amino acid residues such as histidine and methionine of *Ss*ADH cannot be excluded.

The activation of *Ss*ADH by IAA follows a two-step reaction mechanism typical of affinity labeling reactions: the affinity label initially binds to the enzyme and subsequently reacts with the susceptible residue. Similar results were obtained in the modification via a two-step mechanism of Cys 46 in HLADH and Cys 174 in each of the human $\beta\beta$ isoenzymes (Reynolds & McKinley-McKee, 1969; Bosron et al., 1986). As in these cases, the overall rate of *Ss*ADH activation is significantly higher than the uncatalyzed bimolecular model reaction rate (Dahl & McKinley-McKee, 1981a), as a consequence of reversible binding of the IAA molecule to the active site. It is proposed that the carboxylate group of IAA interacts with the imidazole group of His 39, which is adjacent to Cys 38, the site of alkylation, as suggested by the inhibiting effect of chloride and phosphate ions on activation by IAA. The inhibition can be due to the competition of anions for the same anion-binding site, located in the catalytic site of ADHs, as reported by Syversten and McKinley-McKee (1981). In horse liver and other related

ADHs, the anion-binding site is mainly represented by Arg 47, and its guanidinium group plays a specific role in directing carboxymethylation in HLADH (Lange et al., 1975). In *Ss*ADH the anion-binding site has been attributed to His 39, although other residues could be involved (Ammendola et al., 1992; Raia et al., 1994). Preliminary modification experiments with diethyl pyrocarbonate indicate that this specific histidine reagent fully inactivates *Ss*ADH and that NADH protects it. By assuming that the carboxylate group of IAA interacts with the anion-binding site of *Ss*ADH before alkylation can proceed, the presence of anions such as phosphate or chloride can partially prevent the specific interaction. However, simulated computer graphics studies would be required to verify this hypothesis. The higher activation observed with imidazole is consistent with the well-known effect that this molecule shows in increasing the rate and improving the specificity of incorporation of the carboxymethyl group onto Cys 46 of HLADH (Dahl & McKinley-McKee, 1981c; Zeppezauer et al., 1975).

The protection against IAA by a slight excess of NAD^+ is noteworthy, suggesting very strong binding of this ligand to the enzyme. However, the protection does not occur completely under these conditions, since the activity starts increasing about 20 min after the addition of the reagent (Figure 1A). The uncompetitive character of the protection suggests that NAD^+ prefers to bind to the E·A complex rather than to the free enzyme. Furthermore, it seems that this binding facilitates the reversible binding of IAA to the enzyme and slows down the alkylation reaction. Interestingly, an increase in the affinity has been suggested for the binding of NAD^+ to HLADH complexed with 3-bromopropionic acid (Chadha & Plapp, 1984). However, protection experiments with finely tuned NAD^+ concentrations would be required to determine the dissociation constant of the *Ss*ADH·NAD^+ complex in the presence of the affinity label. The partial protection afforded by ADP and ADP−ribose against alkylation by IAA reflects the weaker binding that these coenzyme fragments have to the ADH from horse liver and also the stronger binding of ADP−ribose than ADP (Brändén et al., 1975).

Migration of modified *Ss*ADH as a single band in nondenaturing gel conditions clearly indicates that carboxymethylation was complete and that no partially carboxymethylated species were produced; furthermore, the increased mobility of the modified enzyme, although small, is significant and is consistent with two negative charges introduced per dimeric molecule. Carboxymethylation changes the intrinsic thermostability of this archaeal ADH, rendering it somewhat similar to a mesophilic enzyme. Its dehydrogenase activity, although higher than that of the native enzyme throughout the temperature range investigated, decreases abruptly at 65 °C. Since the change in the CD signal at 222 nm coincides with the sudden drop in activity, the inactivation is a consequence of thermal denaturation. The Arrhenius plot is linear in both cases with similar energy of activation values, suggesting that the different steps of the overall reaction do not become rate-limiting at different temperatures for either native or modified enzymes.

The carboxymethyl group introduced to one of the two cysteine ligands of the catalytic zinc in *Ss*ADH alters the environment of both substrate- and coenzyme-binding sites. The substrate affinity of the modified enzyme decreases significantly more for aliphatic than for aromatic alcohols and more for *meta*- than *para*-substituted benzyl alcohols. This suggests that the carboxymethyl group introduced may be positioned such that it offers more steric hindrance to alkyl groups than to the benzene ring and more steric and electrostatic hindrance to aromatic ring substituents in the *meta* rather than the *para* position.

Furthermore, electronic factors seem to be as determining as steric factors in the change in kinetic behavior produced by carboxymethylation. The mechanism of the well-known horse liver ADH has been investigated extensively (Brändén et al., 1975; Shearer et al., 1993; Czerlinski G.H., 1993) and, in simplified form, can be represented as

$$E \rightleftharpoons E\cdot NAD^+ \rightleftharpoons E\cdot NAD^+\cdot X \rightleftharpoons E\cdot NADH\cdot Y \rightleftharpoons E\cdot NADH \rightleftharpoons E$$

where X and Y represent alcohol and aldehyde/ketone, respectively. The last step of the forward reaction, NADH dissociation, is the slowest and limits the overall reaction rate. The third step, the interconversion of the ternary complex, involves both the release of a proton to the solvent and the transfer of a hydride ion from alcohol to NAD^+, the first process being faster than the latter.

Carboxymethylation of Cys 46 in HLADH causes changes in some of the kinetic properties of the enzyme. The rate of hydride transfer is 700-fold lower for carboxymethylated HLADH and is the rate-limiting step for alcohol oxidation, while the V_{max} value of modified enzyme is about 4% that of the native enzyme (Hardmann, 1976). Data from isotope effect studies indicate that the rate-limiting step is not the same in native and modified *Ss*ADHs. The lack of a significant isotope effect in native enzyme ($V_{max}^H \sim V_{max}^D$) with aliphatic and aromatic alcohols seems to exclude the rate-limiting step being hydride transfer, but that it instead is NADH dissociation, as found for HLADH. On the other hand, for modified *Ss*ADH the isotope effects are large enough to indicate that hydride transfer is rate-limiting. It seems that the rate of the NADH dissociation step is increased considerably upon carboxymethylation and that the steady-state rates of oxidation for the modified enzyme reflect the hydride transfer process. This is seen in the different k_{cat} values for substituted benzyl alcohols, which reflect the character of the substituents in the aromatic ring. The electron-donating groups such as methoxy favor hydride transfer, inductively increasing electron density at the hydride donor carbon C-1, while the electron-withdrawing groups, such as bromine, have the opposite effect. Furthermore, the preference of modified *Ss*ADH for benzyl alcohols with substituents in the *para* rather than the *meta* position agrees with the greater influence that both electron-donating and -withdrawing groups exert in the *para* and *ortho* positions with respect to the *meta* position (Morrison & Boyd, 1992).

The increase in the dissociation rate of NADH is considered to be due to electrostatic repulsion between the negatively charged carboxymethyl group and the pyrophosphate moiety of the coenzyme. This is supported by the double order of magnitude increase in $K_{d(NADH)}$, which reflects a significant weakening of the binary enzyme−coenzyme complex. The binding of NAD^+ is also weakened upon carboxymethylation, as the dissociation constant increased by about 60-fold. As far as the mechanism of the reverse reaction is concerned, we hypothesize that both the decreased acceptance of coenzyme and the influence of the

carboxymethyl group on hydride transfer can affect the kinetic parameters of the overall mechanism, resulting in the decrease in the velocity of aldehyde reduction observed.

The difference in kinetic behavior between archaeal and horse liver ADH upon carboxymethylation is noteworthy, although the mechanism and selectivity of the alkylation reaction are quite similar for both of the enzymes. Interestingly, BrImPpOH, an alkylating derivative of imidazole that totally inactivates HLADH by binding to both the anion-binding site and the catalytic zinc ion (Dahl et al., 1983), has, as in the case of YADH (Dahl & McKinley-McKee, 1977), no effect on *Ss*ADH activity. This is a further indication of subtle differences in the three-dimensional structure of the catalytic site of various dimeric and tetrameric zinc alcohol dehydrogenases.

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