Frieden, C., and Fernandez Sousa (1975), *J. Biol. Chem. 250,* 2106.
Gerding, R. K., and Wolfe, R. G. (1969), *J. Biol. Chem. 244,* 1164.
Harada, K., and Wolfe, R. G. (1968), *J. Biol. Chem. 243,* 4131.
Holbrook, J. J. (1972), *Biochem. J. 128,* 921.
Holbrook, J. J., and Wolfe, R. G. (1972), *Biochemistry 11,* 2499.
Holbrook, J. J., Yates, D. W., Reynolds, S. J., Evans, R. W., Greenwood, C., and Gore, M. G. (1972), *Biochem. J. 128,* 933.
Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365.
Lazdunski, M., Petitcherc, C., Chappelet, D., and Lazdunski, C. (1971), *Eur. J. Biochem. 20,* 124.
Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 1121.
Levitzki, A., and Koshland, D. E., Jr. (1972), *Biochemistry 11,* 241.
Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr. (1971), *Biochemistry 10,* 3371.
Long, C. W., Levitzki, A., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245,* 80.
Malhotra, O., and Bernhard, S. A. (1968), *J. Biol. Chem. 243,* 1243.
Malhotra, O., and Bernhard, S. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2077.
Pfleiderer, G., and Auricchio, F. (1964), *Biochem. Biophys. Res. Commun. 16,* 53.
Simpson, R. T., and Vallee, B. L. (1970), *Biochemistry 9,* 953.
Thorne, C. J. R., and Kaplan, N. O. (1963), *J. Biol. Chem. 238,* 1861.
Tsernglou, D., Hill, E., and Banazak, L. J. (1972), *J. Mol. Biol. 69,* 75.

The Role of Arginyl Residues in Directing Carboxymethylation of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The selective carboxymethylation by iodoacetate of Cys-46 in the active center of horse liver alcohol dehydrogenase has been shown to be mediated by interaction of the anionic reagent with the arginyl residue(s) previously shown to be responsible for binding NADH (L. G. Lange, J. F. Riordan, and B. L. Vallee (1974), *Biochemistry 13,* 4361). Thus, sequential and reversible chemical modification of arginine with butanedione and of cysteine with *p*-mercuribenzoate demonstrate that the essential thiol groups are not affected by arginine modification. Importantly, the rate of incorporation of [^{14}C]iodoacetate into native horse liver alcohol dehydrogenase is ten times faster than that for the butanedione-modified enzyme. Moreover, as evidenced by peptide isolation, the radiolabel incorporated into the latter occurs at low levels in several different peptides as opposed to the single, strongly labeled CmCys-46 peptide obtained from the native enzyme. The demonstration that the arginyl residue(s) involved in coenzyme binding promotes enhanced reactivity of the active site thiol supports the general hypothesis that the spatial arrangement of structural features allowing expression of enzymatic function may also account for enhanced chemical reactivity of certain active site residues (B. L. Vallee and J. F. Riordan (1969), *Annu. Rev. Biochem. 38,* 733).

Selective carboxymethylation of horse liver alcohol dehydrogenase with iodoacetate has demonstrated the existence of a catalytically essential, active site cysteine residue (Li and Vallee, 1964) later shown to be Cys-46 (Jörnvall, 1970). Only iodoacetate reacts rapidly and specifically. Iodoacetamide, for example, reacts slowly and nonselectively (Li and Vallee, 1965). This observation suggests that a negatively charged carboxylate group might be instrumental in directing the reagent to the active site. Indeed, Reynolds and McKinley-McKee (1969) and Reynolds et al. (1970) have shown that prior to irreversible alkylation iodoacetate and enzyme form a reversible complex. NADH, ADPR,[1] and other anionic ligands such as chloride prevent this interaction competitively, consistent with the presence of a cationic site near Cys-46 which might bind both the coenzyme and the carboxylate group of iodoacetate. Further, the anion $Pt(CN)_4^{2-}$ employed in the preparation of isomorphous heavy atom derivatives of the enzyme for X-ray structure analysis inhibits horse liver alcohol dehydrogenase by competition with coenzyme (Gunnarsson et al., 1974) consistent with the existence of such a site.

Recently chemical modifications have shown that arginyl residues serve as NADH binding sites of the alcohol dehydrogenases from human and horse liver and from yeast (Lange et al., 1974). These results are entirely in accord with the results of X-ray structure analysis of the horse liver enzyme (Brändén et al., 1975). We have now employed se-

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[1] Abbreviations used are: ADPR, adenine diphosphoribose; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

quential chemical modifications of arginine and cysteine to identify the anion binding site likely responsible for directing the specific carboxymethylation of Cys-46 by iodoacetate. The data strongly indicate that this site is an arginine residue.

Materials and Methods

Horse liver alcohol dehydrogenase (Boehringer-Mannheim Corp.) was obtained as a crystalline suspension in 0.02 *M* phosphate buffer (pH 7.5) and 10% ethanol. Concentrated enzyme solutions were prepared by dialyzing the suspension for 3 days vs. daily changes of a 1000-fold volume excess of 0.1 *M* phosphate buffer (pH 7.5), 4°; centrifugation at 5000 rpm for 15 min, 4°, removed insoluble material. Protein concentrations, using a molar absorptivity at 280 nm of $3.57 \times 10^4\ M^{-1}\ cm^{-1}$, and enzymatic activity, which varied from 12.9 to 13.6 ΔA_{340} per min per mg, were determined by the method of Drum et al. (1969).

The oxidized and reduced forms of β-diphosphopyridine nucleotide from yeast were obtained from Sigma. Solutions were prepared daily and kept on ice. The sources of 2,3-butanedione and *p*-mercuribenzoate were Aldrich and Sigma Chemical Companies, respectively.

Essential sulfhydryl groups were protected by adding 1 m*M* *p*-mercuribenzoate in 50 m*M* borate, pH 8.5, 25°, to horse liver alcohol dehydrogenase, 4.82 mg/ml, in the same buffer. This product was subjected to arginine modification by incubating it for 1 hr at 25° with an appropriate aliquot of 116 m*M* butanedione in borate. Unreacted butanedione was then removed by dialysis for 2–3 hr vs. a 1000-fold volume excess of 50 m*M* borate, pH 8.5, 4°, and without affecting the activity of the enzyme. To regenerate free sulfhydryl groups, concentrated dithiothreitol was added to give a final concentration of 2 m*M*.

Conversely, horse liver alcohol dehydrogenase, 4.82 mg/ml, was incubated first in 5 m*M* butanedione in 50 m*M* borate (pH 8.5). The resulting derivative was then treated with 0.36 m*M* *p*-mercuribenzoate in borate (pH 8.5) and gel filtered within 5 min through Bio-Gel P-4 equilibrated with 20 m*M* Veronal (pH 8.5). In a control experiment, enzymatic activity was regenerated by gel filtration in Veronal but without prior reaction with *p*-mercuribenzoate.

Horse liver alcohol dehydrogenase, 0.71 mg/ml, in 50 m*M* borate (pH 8.5) was carboxymethylated with 5.1 m*M* iodoacetate-*1*-^{14}C (198,000 cpm/μmol, New England Nuclear) at 20° in the presence and absence of 20 m*M* $K_2Pt(CN)_4$ according to the procedure of Li and Vallee (1965). Aliquots were removed at intervals and assayed for enzymatic activity and ^{14}C-incorporation, the latter after gel filtration through Bio-Gel P-4 equilibrated with 50 m*M* borate. The same procedure was employed for the butanedione-modified enzyme.

Peptide Isolation. The carboxymethylated butanedione derivative of liver alcohol dehydrogenase was prepared by first incubating the native enzyme, 0.83 mg/ml, in 50 m*M* borate (pH 8.5) with 5 m*M* butanedione at 20°. After 54 min the resultant product was gel filtered through Bio-Gel P-4 in borate and separated from unreacted butanedione. This species was then carboxymethylated with 5.1 m*M* [^{14}C]iodoacetate, a 600-fold molar excess. After 90 min the reaction mixture was gel filtered as above, except in 20 m*M* Veronal (pH 8.5). The removal of borate allows the spontaneous reversal of the arginine modification. Aliquots of the appropriate eluate fractions were assayed both for ^{14}C-incorporation and enzymatic activity. Half of the material was processed further for peptide isolation (vide infra). The other half, following dialysis at 4° for 3 hr vs. a 200-fold volume excess of 50 m*M* borate (pH 8.5), was again treated with a 600-fold molar excess of [^{14}C]iodoacetate (4.79 m*M*). After 90 min, it was gel filtered in NH_4OH (pH 8.3) and processed for peptide isolation.

In a parallel experiment, a control sample of horse liver alcohol dehydrogenase, 0.83 mg/ml, in 50 m*M* borate (pH 8.5) was gel filtered in borate after standing at 20° for 54 min and then inactivated by the addition of [^{14}C]iodoacetate (5.1 m*M*) in borate. The resultant carboxymethyl alcohol dehydrogenase was separated from excess reagent by gel filtration in 20 m*M* Veronal (pH 8.5). Half of this material was digested with trypsin (vide infra) and the remainder, after dialysis at 4° for 3 hr vs. a 200-fold volume excess of borate, was treated with a 600-fold molar excess of [^{14}C]iodoacetate for 90 min. It was then gel filtered in NH_4OH (pH 8.3) and subjected to tryptic digestion.

In all cases, the ^{14}C-carboxymethylated peptides resulting from tryptic digestion were isolated according to the procedure of Li and Vallee (1964). After removal of unreacted [^{14}C]iodoacetate by gel filtration through Bio-Gel P-4, equilibrated with NH_4OH (pH 8.3), the sample was incubated with 1% by weight TPCK-trypsin (Worthington) at 37° for 60 min. Another 1% trypsin was then added and digestion continued for another 60 min. The mixture was evaporated to dryness at 30° under vacuum pump pressure and then dissolved in 0.5 ml of NH_4OH–0.2% thiodiglycol (pH 8.3). The ^{14}C-labeled peptides were chromatographed over Sephadex G-200 superfine (15 × 25 cm) equilibrated with the same buffer. The flow rate was 6.7 ml/hr and 2-ml fractions were collected.

The resulting ^{14}C-labeled peptides were pooled, evaporated to dryness, dissolved in 0.5 ml of 0.17 *M* pyridine acetate–0.2% thiodiglycol (pH 4.7) and applied to a Bio-Rad cation exchange resin, AG 50w-X2 (0.9 × 24 cm), equilibrated, and developed with the same buffer. Fractions, 1.4 ml, were collected at a flow rate of 14 ml/hr and assayed both for ^{14}C and for ninhydrin positive material by the procedure of Moore and Stein (1954). Amino acid analyses were carried out on samples hydrolyzed in 6 *N* HCl at 110° in sealed, evacuated ampoules for 24 and 72 hr using a Durrum 500 analyzer.

Results

Butanedione Modification of Mercuribenzoate–Liver Alcohol Dehydrogenase. At pH 8.5 in 50 m*M* borate, *p*-mercuribenzoate inactivates horse liver alcohol dehydrogenase virtually instantaneously to levels of activity determined by reagent concentration. Activity can be regenerated by dithiothreitol, 2 m*M*, to an extent dependent on the previous degree of inactivation. Thus, alcohol dehydrogenase inactivated with *p*-mercuribenzoate to 10% of the activity of the native enzyme can be reactivated with dithiothreitol to 40% and that inactivated to 22% can be reactivated to 72%.

The maximal degree of inactivation which still allows subsequent reactivation to 100% of native activity is 66%, obtained as a result of exposing the enzyme to 0.36 m*M* *p*-mercuribenzoate in 50 m*M* borate (pH 8.5). Hence, this concentration was selected for reversible protection of sulfhydryl groups (Figure 1). Horse liver alcohol dehydrogenase, inactivated under these conditions, is stable for at least 30 min at 25°. Moreover, after dialysis against borate at 4° for 3 hr, it can be fully reactivated within 30 min. On the other hand, treatment of this 34% active species with 5

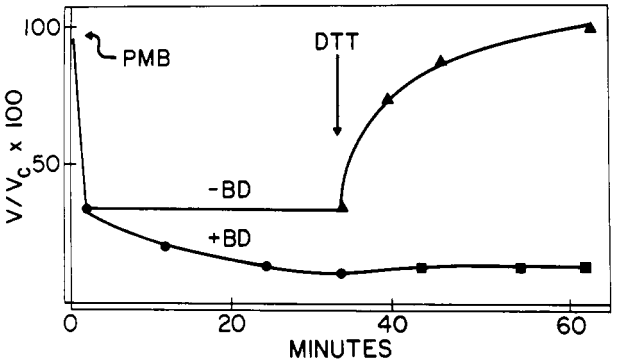

FIGURE 1: Changes in activity on incubating horse liver alcohol dehydrogenase, 4.82 mg/ml, with 0.36 m*M* *p*-mercuribenzoate (PMB) in 50 m*M* borate, pH 8.5, 25°. One aliquot (▲) was further incubated in borate for 30 min followed by addition of concentrated dithiothreitol (DTT, arrow) to give a final concentration of 2 m*M* DTT. A second aliquot (●) was made 5 m*M* in butanedione (BD) and allowed to incubate at 25° for 30 min. Concentrated DTT was then added (arrow) and at intervals aliquots were removed and assayed for enzymatic activity (■).

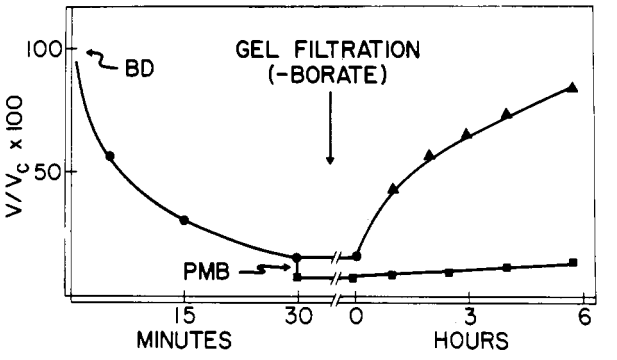

FIGURE 2: Changes in activity on incubating horse liver alcohol dehydrogenase, 4.82 mg/ml, with 5 m*M* butanedione (BD) in 50 m*M* borate (pH 8.5) at 25°. After 30 min one aliquot (● and ▲) was gel filtered (arrow) through Bio-Gel P-4 equilibrated with 20 m*M* Veronal (pH 8.5) to remove borate. A second aliquot (■) was treated with 0.36 m*M* *p*-mercuribenzoate (PMB) in borate (pH 8.5) and then gel filtered through Bio-Gel P-4 in Veronal. In each case aliquots of the appropriate eluate fraction were removed at intervals and assayed for enzymatic activity.

m*M* butanedione in 50 m*M* borate (pH 8.5) for 30 min further reduces activity to 9% of the native enzyme. After dialysis at 4° against borate for 3 hr, addition of dithiothreitol has little effect. Only 14% of the native activity is present after 30 min of exposure. When dithiothreitol is added to butanedione-modified liver alcohol dehydrogenase serving as a control, there is no reactivation.

Mercuribenzoate Modification of Butanedione-Treated Liver Alcohol Dehydrogenase. Modification of horse liver alcohol dehydrogenase with butanedione in borate (pH 8.5) for 30 min selectively modifies the active site arginyl residues responsible for NADH binding (Lange et al., 1974). The resulting product is stable but 90% of the native activity is recovered on removal of borate by gel filtration in 20 m*M* Veronal (pH 8.5) (Figure 2). However, progressively less activity can be regained if the butanedione modification reaction is allowed to proceed for more than 60 min.

Addition of 0.36 m*M* *p*-mercuribenzoate to butanedione-treated liver alcohol dehydrogenase exhibiting 18% of the native activity further reduces activity to 5% within a few seconds. Now, however, gel filtration in Veronal reactivates the modified enzyme to only 15% of the native activity (Figure 2, lower curve).

Carboxymethylation of Native and Butanedione-Treated Liver Alcohol Dehydrogenase. Treatment of native horse liver alcohol dehydrogenase with a 600-fold molar excess of iodoacetate in 50 m*M* borate (pH 8.5) rapidly inactivates the enzyme due to carboxymethylation of two cysteinyl SH groups per dimer (Li and Vallee, 1964). Using labeled iodoacetate, under these conditions, the $t_{1/2}$ of ^{14}C-incorporation is 11 min (Figure 3). The rate of carboxymethylation was also determined for a sample of butanedione-treated liver alcohol dehydrogenase having 14% residual activity. In marked contrast to the native enzyme, the rate of reaction is nearly an order of magnitude slower ($t_{1/2}$ = 100 min).

$K_2Pt(CN)_4$ prevents both inactivation of native liver alcohol dehydrogenase by [^{14}C]iodoacetate and ^{14}C-incorporation. Modification of the native enzyme, 0.83 mg/ml, with a 600-fold molar excess of [^{14}C]iodoacetate for 90 min in the presence of 20 m*M* $K_2Pt(CN)_4$ only produces a loss of 2% of the native activity and a concomitant incorporation of only 0.14 [^{14}C]carboxymethyl group per dimer of enzyme. By contrast, in the absence of $K_2Pt(CN)_4$, the native enzyme is inactivated 85% and 2.16 [^{14}C]carboxymethyl groups per dimer are incorporated.

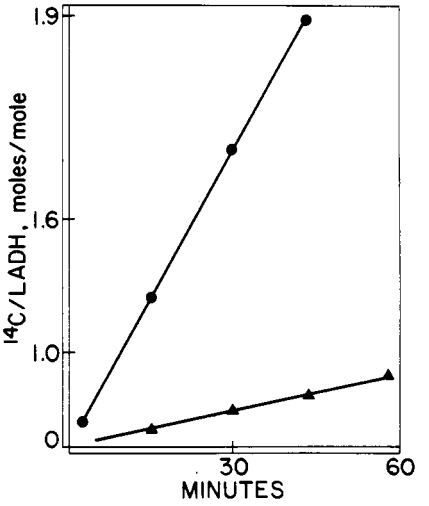

FIGURE 3: Time course for the incorporation of ^{14}C in native (●) and butanedione-modified (▲) liver alcohol dehydrogenase when incubated with iodoacetate-^{14}C. Native or butanedione-modified liver alcohol dehydrogenase, 0.71 mg/ml, in 50 m*M* borate (pH 8.5) was carboxymethylated with 5.1 m*M* iodoacetate-^{14}C. Aliquots were removed at intervals and assayed for activity and ^{14}C-incorporation, the latter after gel filtration through Bio-Gel P-4 equilibrated with 50 m*M* borate (pH 8.5).

Carboxymethylated Peptides from Native and Butanedione-Treated Liver Alcohol Dehydrogenase. Li and Vallee (1964) found that carboxymethylation of native liver alcohol dehydrogenase, 0.7 mg/ml, with a 600-fold molar excess of [^{14}C]iodoacetate inactivates the enzyme (Figure 4). These experiments were repeated on native and butanedione-modified alcohol dehydrogenase (Figure 4, arrow). For the native enzyme, and as previously shown, loss of activity is virtually complete within 60 min (Figure 4, triangles) and correlates with the incoporation of 2.2 [^{14}C]carboxymethyl groups/molecule of enzyme. As a control, treatment of this sample with an additional 600-fold molar excess of [^{14}C]iodoacetate for 90 min results in an increase in the degree of substitution to 2.3 [^{14}C]carboxymethyl groups/molecule, an increase of only 0.1 group. Another

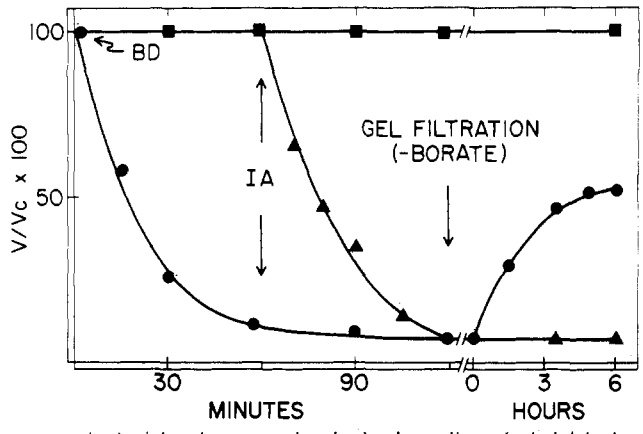

FIGURE 4: Activity changes on incubating horse liver alcohol dehydrogenase, 0.83 mg/ml, with 50 m*M* borate (pH 8.5) (■) and with 5 m*M* butanedione (BD) in 50 m*M* borate (●). After 60 min and following gel filtration in borate (not shown) each sample, 0.71 mg/ml, was treated, first arrow, with 5.1 m*M* iodoacetate-^{14}C (IA) for another 60 min (▲, ●) and subsequently gel filtered through Bio-Gel P-4 in 20 m*M* Veronal (pH 8.5), second arrow. Aliquots of the appropriate eluate fraction were removed at intervals and assayed for enzymatic activity. The unmodified control activity is shown at the top throughout (■).

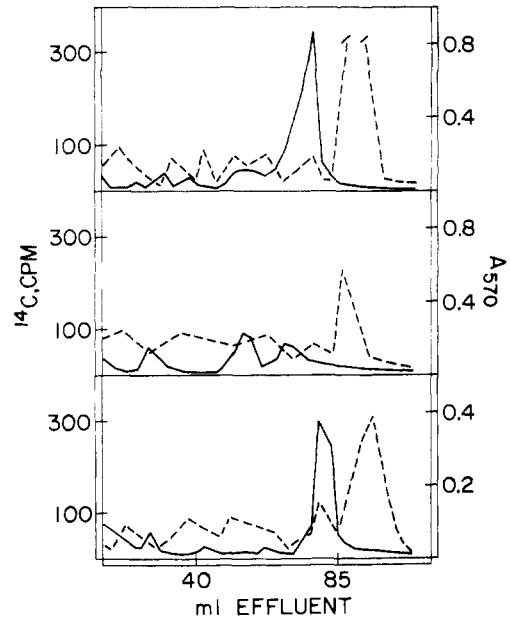

FIGURE 5: (Top) Chromatographic elution profile of peptides resulting from native liver alcohol dehydrogenase, 0.71 mg/ml, which had been carboxymethylated with 5.1 m*M* iodoacetate-^{14}C in 50 m*M* borate, digested with trypsin, and then fractionated on Sephadex G-200. Complete digestion was indicated by the absence of any large molecular weight material. The digest (see Materials and Methods) was dissolved in 0.17 *M* pyridine acetate–0.2% thiodiglycol, (pH 4.7) and applied to a Bio-Rad AG 50w-X2 (0.9 × 24 cm) column equilibrated and developed with the same buffer. Fractions, 1.4 ml, were collected at a flow rate of 14 ml/hr and assayed for both radioactivity (—) and ninhydrin positive material (Moore and Stein, 1954) (- - -). (Middle) Chromatographic elution profile of peptides resulting from butanedione-modified liver alcohol dehydrogenase, 0.71 mg/ml, which was subsequently carboxymethylated with 5.1 m*M* iodoacetate-^{14}C in 50 m*M* borate (pH 8.5), gel-filtered through Bio-Gel P-4 in 20 m*M* Veronal, and then digested with trypsin. All other conditions were as described above. (Bottom) Chromatographic elution profile of peptides from liver alcohol dehydrogenase prepared as above (middle) except that, following gel filtration in Veronal, the enzyme was carboxymethylated a second time with a 600-fold molar excess of iodoacetate-^{14}C. Other conditions were as already described. The large 570-nm absorbing peak centered near 105 ml represents ammonia removed incompletely during rotatory evaporation and which serves as an additional marker.

sample of enzyme was inactivated to 14% of native activity by reaction with butanedione for 60 min (Figure 4, circles). Treatment of this material with a 600-fold molar excess of [^{14}C]iodoacetate (Figure 4, arrow) results in the incorporation of 0.85 mol of radiolabel/mol of enzyme. Removal of borate from this species by gel filtration (Figure 4, arrow) reverses arginine modification (Riordan, 1973) and concomitantly restores 50–60% of native activity. If this sample is recarboxymethylated with a second 600-fold molar excess addition of [^{14}C]iodoacetate, rapid inactivation to 8% native activity ensues (not shown) and a further 0.90 [^{14}C]carboxymethyl group is incorporated per molecule of enzyme. This additional amount of ^{14}C-incorporation together with the resultant decrease in activity from 50–60% down to 8% is consistent with the stoichiometry of one carboxymethylcysteine per subunit obtained with the native enzyme.

Trypsin digestion of *S*-carboxymethyl alcohol dehydrogenase containing 2.2 [^{14}C]carboxymethyl groups/molecule, followed by gel filtration through Sephadex G-200 superfine and chromatography of the pooled ^{14}C-containing fractions on AG 50w-X2 gave a single peptide accounting for most of the ^{14}C-label (Li and Vallee, 1964). We have repeated this experiment (Figure 5, top) under slightly different conditions using our preparation of [^{14}C]carboxymethyl alcohol dehydrogenase and confirm that essentially all of the radioactivity elutes from the ion-exchange column as a single peak at 76 ml. The amino acid composition of the peptide representing most of the labeled material was determined at 24 and 72 hr of hydrolysis. In both cases, there was found 1 mol of Arg, CmCys, Thr, Gly, Ala, Val, Met, and Ile per mol of radioactive label in accord with the earlier data (Li and Vallee, 1964). This composition is uniquely compatible with the segment of horse liver alcohol dehydrogenase containing residues 40–47 (Jörnvall, 1970).

The chromatographic elution profile of the [^{14}C]carboxymethyl peptides from a sample of butanedione modified, [^{14}C]iodoacetate-treated liver alcohol dehydrogenase, which had been gel filtered to remove borate and hence reverse arginine modification prior to trypsin digestion (Figure 5, middle) differs markedly from that of the carboxymethylated native enzyme. There are small peaks at 25, 52, and 66 ml and several smaller peaks elsewhere. However, the large peak, previously observed at 76 ml in the pattern of the native enzyme, is virtually absent.

Finally, digestion of the recarboxymethylated species containing the additional 0.90 [^{14}C]carboxymethyl group/molecule followed by chromatography on AG 50w-X2 produces a chromatogram which again has the prominent peak at 76 ml (Figure 5, bottom) in addition to the other minor peaks.

Discussion

Active center residues of enzymes often exhibit markedly enhanced reactivity when compared to their counterparts elsewhere in the protein. Clearly, the native, three-dimensional structure is critical both for the expression of potential enzymatic activity and of this enhanced chemical reactivity (Vallee and Riordan, 1969). This relationship, in part, underlies numerous studies (Shaw, 1970; Baker, 1967) in which reagents are designed to mimic substrates and, hence, to become endowed with an intrinsic affinity for the active site of the enzyme in question. While in these cases

the nature of the enhanced reactivity is apparent, certain reagents, not considered to be quasi-substrates based on available knowledge, have been found exceptionally reactive and quite specific for a particular active site residue. It has proven difficult to establish the basis for such enhanced reactivity, but several systems have implicated charge complementarity as an important factor. For example, the phosphate moiety of pyridoxal phosphate has been thought to direct Schiff base formation in glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, and ribonuclease A (Anderson et al., 1966; Rippa et al., 1967; Raetz and Auld, 1972). Further, the positively charged alkylating reagent, α-*N*-bromoacetylarginine methyl ester, is directed toward histidine following tetrathionate protection of the active site thiol of Streptococcal proteinase (Liu, 1967). Also, the iodoacetate alkylations of RNase A and T are known to be affected markedly by neighboring charge interactions (Crestfield et al., 1963; Takahashi et al., 1963).

The present work shows that selective carboxymethylation by iodoacetate of Cys-46 in the active center of horse liver alcohol dehydrogenase is also mediated by charge complementarity. In this case, the anion binding site is an arginyl residue, whose guanidinium side chain would seem to interact with the carboxylate moiety of iodoacetate. Selective reversible arginine modification (Lange et al., 1974) blocks the positively charged guanidinium group and thus precludes its interaction with the carboxylate group of iodoacetate. This then prevents carboxymethylation of Cys-46 (Figures 4 and 5).

This experimental approach implies that butanedione, the reagent used for arginine modification, does not simultaneously modify cysteinyl residues. In fact, the *reversible* modifications of arginine and cysteine here employed have excluded this possibility. Thus, removal of borate from butanedione modified liver alcohol dehydrogenase returns 85–90% of the native activity due to reversal of arginine modification (Riordan, 1973). In contrast, treatment of the same enzyme with mercuribenzoate prior to removal of borate restores very little activity (Figure 2) suggesting that in the arginine modified enzyme the thiol groups remain free to react with mercuribenzoate. This interpretation is consistent with the effect of butanedione on the mercuribenzoate derivative, since the thiol groups are not essential for butanedione to inactivate horse liver alcohol dehydrogenase (Figure 1).

The protection by butanedione against both ^{14}C-incorporation and inactivation by iodoacetate (Figures 3 and 4) provides evidence that arginine is essential in directing the alkylating agent to Cys-46. Prior butanedione modification of liver alcohol dehydrogenase reduces the rate of ^{14}C-incorporation by an order of magnitude compared to the unmodified native enzyme. Any alkyl groups that are incorporated under these conditions do not impair restoration of enzymatic activity by removal of borate to reverse arginine modification; hence the alkylation does not reflect modification of Cys-46.

The peptide isolation data indeed show that in the butanedione-modified enzyme Cys-46 is not carboxymethylated significantly (Figure 5 middle). As shown previously by Li and Vallee (1964) iodoacetate inactivation of horse liver alcohol dehydrogenase correlates with incorporation of two carboxymethyl groups per dimer. Tryptic digestion and chromatography of the resulting peptides on a cation exchange resin produced a single peak of radioactivity corresponding to a single peptide containing CmCys-46. This experiment was repeated (Figure 5, top) and a single peak marking the position of the CmCys-46 peptide was found. On the other hand, the chromatographic separation of peptides derived from liver alcohol dehydrogenase which had first been treated with butanedione and then with [^{14}C]iodoacetate (Figure 5b) revelaed virtually a total absence of any ^{14}C-labeled peptide in the position normally occupied by that containing CmCys-46. Instead, small amounts of the radiolabel were detected in several other positions but without any single prominent peak.

Since tryptic digestion was employed, it was essential to demonstrate that arginine modification was indeed reversible and that the absence of the radiolabel from the position of CmCys-46 was not due to the failure of trypsin to cleave the Arg-47–Ser-48 bond. Successful deblocking of arginine is indicated by the restoration of activity to liver alcohol dehydrogenase treated sequentially with butanedione and then iodoacetate upon removal of borate (Figure 4, second arrow). Equally important is the third chromatograph (Figure 5 bottom) which depicts the peptides resulting from recarboxymethylation of this enzyme. The appearance of radiolabel in the position characteristic for CmCys-46 shows that the arginines responsible for directing carboxymethylation are, indeed, again free and that trypsin has cleaved the adjacent bond. Jointly, these experiments demonstrate that an intact arginyl residue is essential for selective carboxymethylation of Cys-46 by iodoacetate. The enhanced reactivity of this thiol in horse liver alcohol dehydrogenase would seem to be mediated by a charge interaction of the reagent with another active center residue, arginine, that serves as an anion binding site. From the tertiary structure of the enzyme it is possible to deduce a strong candidate for this arginine residue. Eklund et al. (1975) have shown that the general anion binding site in horse liver alcohol dehydrogenase coincides with the site that binds the pyrophosphate moiety of the coenzyme. The center of this site is only 8 Å from the sulfur atom of Cys-46. Furthermore, this sulfur atom is accessible from the direction of the anion binding site but not from any other direction. The only positively charged residue involved in this site is Arg-47. From the structural point of view it is thus quite feasible that the negatively charged end of the iodoacetate molecule is positioned by interaction with the side chain of Arg-47 and that the reactive end of iodoacetate thereby is positioned for chemical attack of Cys-46. No other cysteine residues are accessible from this site within reasonable distance. Identification of the modified arginine residues in the primary sequence is very important to test the validity of these structural deductions. Work on this problem is in progress.

This neighboring group interaction probably has even more general significance than could have been surmised since it has been learned subsequently that Cys-46 is one of the protein ligands to the active site zinc ion (Eklund et al., 1974). The spatial relations of the anion binding site, Arg-47, Cys-46, and the active site zinc atom are schematically illustrated in Figure 6. Thus owing to this proximity, not only does arginine direct attack to a particular cysteine which is a ligand, but it also renders it susceptible to alkylation while actually bound to zinc.

It has proven exceptionally difficult to identify metal ligands of metalloenzymes by chemical means; even so, methodologically the use of metals as differential labels in these systems has been accepted without much experimental support, based largely on analogous experiments employing coenzymes, substrates, or inhibitors. The present

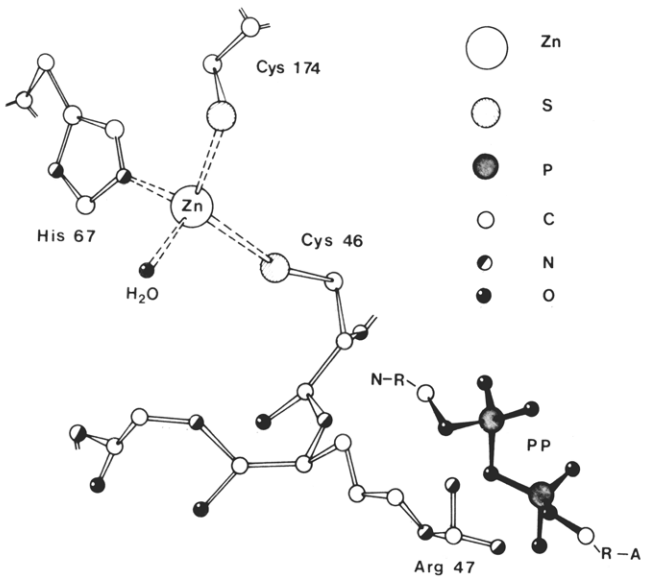

FIGURE 6: Diagram illustrating the spatial relationship of the active site zinc atom, Cys-46, Arg-47, and the anion binding site of horse liver alcohol dehydrogenase as found in the X-ray studies (Eklund et al., 1974). The anion binding site is illustrated by the position, marked PP, of the pyrophosphate moiety of ADP-ribose, when bound to liver alcohol dehydrogenase (Abdallah et al., 1975). R-A and R-N denote the adenosine end and terminal ribose end, respectively, of the nucleotide.

data suggest that the validity of such an approach may depend on the role(s) of other residues in the active center. Thus, for example, while a sulfhydryl group may be less susceptible to attack when bound to a metal, its overall reactivity may be enhanced owing to interaction of a neighboring group, e.g., an arginyl residue, with the reactive species, e.g., iodoacetate. The occurrence of such systems and their importance to identifying metal binding sites may be more common than presently appreciated. It would appear that the present instance represents a novel form of affinity labeling affecting a metal ligand directed by a coenzyme binding residue.

References

Abdallah, M. A., Biellman, J.-F., Nordström, B., and Brändén, C.-I. (1975), *Eur. J. Biochem. 50,* 475.

Anderson, B. M., Anderson, C. D., and Churchich, J. E. (1966), *Biochemistry 5,* 2893.

Baker, B. R. (1967), Design of Active-Site-Directed Irreversible Inhibitors, New York, N.Y., Wiley.

Brändén, C.-I., Jörnvall, H., Eklund, H., and Furugren, B. (1975), *Enzymes, 3rd Ed.* (in press).

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238,* 2413.

Drum, D. E., Li, T.-K., and Vallee, B. L. (1969), *Biochemistry 8,* 3783.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. and Brändén, C.-I. (1974), *FEBS Lett. 44,* 200.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B.-O., Tapia, O., Brändén, C.-I., and Åkeson, Å (1975), *J. Mol. Biol.* (in press).

Gunnarsson, P.-O., Pettersson, G., and Zeppezauer, M. (1974), *Eur. J. Biochem. 43,* 479.

Jörnvall, H. (1970), *Eur. J. Biochem. 16,* 25.

Lange, L. G., Riordan, J. F., and Vallee, B. L. (1974), *Biochemistry 13,* 4361.

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

Li, T.-K., and Vallee, B. L. (1965), *Biochemistry 4,* 1195.

Liu, T.-Y. (1967), *J. Biol. Chem. 242,* 4029.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211,* 907.

Raetz, C. H. R., and Auld, D. S. (1972), *Biochemistry 11,* 2229.

Reynolds, C. H., and McKinley-McKee, J. S. (1969), *Eur. J. Biochem. 10,* 474.

Reynolds, C. H., Morris, D. L., and McKinley-McKee, J. S. (1970), *Eur. J. Biochem. 14,* 14.

Riordan, J. F. (1973), *Biochemistry 12,* 3915.

Rippa, M., Spanio, L., and Pontremoli, S. (1967), *Arch. Biochem. Biophys. 118,* 48.

Shaw, E. (1970), *Enzymes, 3rd Ed. 7,* 91.

Takahashi, K., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238,* 2413.

Vallee, B. L., and Riordan, J. F. (1969), *Annu. Rev. Biochem. 38,* 733.