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# IDENTIFICATION OF THE THIOL RESIDUES INVOLVED IN MODIFICATIONS OF PIG HEART LIPOAMIDE DEHYDROGENASE BY CUPRIC ION AND BY IODOACETAMIDE

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## SUMMARY

The thiol residues involved in two previously described modifications of heart lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) have been identified by comparison of peptide maps of unmodified and modified enzymes. Two thiols and one methionine react when native enzyme is alkylated in concentrated iodoacetamide, with the accompanying loss of enzymatic activity and an NAD-binding site. NAD protects the more slowly reacting thiol from alkylation, and the NAD-protected enzyme is active and retains its NAD-binding site. Loss of the binding site, and loss of activity are associated with the alkylation of two neutral thiol peptides which may represent alternative versions of a single thiol region in the enzyme. Treatment of native enzyme with cupric ion results in the rapid oxidation of two thiols to a disulfide bond and loss of NADH-lipoamide reductase activity. We have determined that thiol residues in the cationic peptide and one of the anionic peptides are involved in the disulfide bond formed by cupric ion. Since the cationic peptide contains two histidyl residues, it is proposed that it is the initial site of binding of cupric ion, prior to the oxidation of the thiol residues to a disulfide bond.

## INTRODUCTION

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) accepts and donates 2 electrons in its catalytic cycle [1]. Since the enzyme contains both FAD and a redox active disulfide, it can accept 4 electrons under various forcing conditions. Reduction in the presence of arsenite leads to 4-electron reduction (and to inactivation in the lipoate reaction) due to the formation of a covalent adduct between the vicinal dithiol and the arsenite. If this reduction has been effected with NADH, resulting in the formation of NAD, the spectrum of the reduced enzyme displays a broad long wavelength absorption centering around 700 nm due to charge-transfer interaction between $FADH_2$ and NAD [2, 3].

Stein and Stein [4] have shown that alkylation of native pig heart lipoamide

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

dehydrogenase by concentrated iodoacetamide is associated with a 95% decrease in the NADH-lipoamide reductase activity of the enzyme and with loss of the ability to form the 700 nm charge-transfer band. Since the formation of this band is absolutely dependent on the presence of pyridine nucleotide, they interpreted their results as indicating that the alkylation had destroyed the NAD-binding site by reaction with a thiol. They did not, however, demonstrate that a thiol was the site of alkylation. They indicated that the alkylated enzyme could be reduced to the red 2-electron intermediate by 1 equivalent of NADH.

Treatment of native enzyme with cupric ion also leads to a 90–95% reduction in the NADH-lipoamide reductase activity of the enzyme. Although this enzyme is also capable of reduction by either NADH or reduced lipoamide these proceed at a much slower rate [5]. Veeger and Massey [5] and Casola et al. [6] have presented data indicating that the inactivation of the enzyme involves the formation of a new disulfide bond from two of the thiols of native enzyme.

Since the enzyme contains seven to eight thiol groups per mole of enzyme bound FAD [7] we wished to be able to distinguish those thiol groups which were modified by treatment of heart lipoamide dehydrogenase with cupric ion, and by alkylation of the native enzyme in concentrated iodoacetamide. In this way we hoped to reach a clearer understanding of the role of thiols in the catalytic activity of the enzyme. A preliminary report on some of the work presented here has appeared [8].

## MATERIALS AND METHODS

The methods for preparation of pig heart lipoamide dehydrogenase, for 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) titrations of enzyme thiol groups, for anaerobic experiments, for alkylation and digestion of the denatured enzyme, and for the preparation of two-dimensional peptide maps, as well as the peptide numbering system are given previously [7].

Conditions for amino acid analysis of samples of modified enzyme were the same as those described previously [7] for amino acid analysis of peptides, except that $\beta$-mercaptoethanol was not added to the 6 M HCl used to digest samples of whole protein. Alkylated methionine was estimated by summing the areas under the peaks for *S*-carboxymethylhomocysteine, homoserine and homoserine lactone [9].

NADH-lipoamide reductase assays were performed essentially as described by Massey [10] using 3 ml vol. containing 0.05 M phosphate buffer, pH 6.3; bovine serum albumin, 2 mg; NADH 0.3 $\mu$mole; NAD, 0.2 $\mu$mole; D,L-lipoamide, 2 $\mu$moles.

*Inactivation of pig heart lipoamide dehydrogenase by cupric ion*

The enzyme (100 nmoles/ml) was dialyzed against two 1-l changes of glass-distilled, deionized water to remove all EDTA and buffer. The dialyzed enzyme was brought to 0.03 M in phosphate buffer, pH 7.6. An 8-fold excess of cupric sulfate over enzyme-bound FAD was added, and the solution incubated on ice for 30 min. A 10-fold excess of neutralized EDTA over copper sulfate was then added to quench the reaction, and the copper–EDTA complex was separated from the enzyme by passage over a Sephadex G-25 column equilibrated with 0.1 M phosphate (pH 7.6)–0.4 mM EDTA. On addition of cupric ion to the enzyme solution, a light precipitate forms, indicating that the solubility product for cupric phosphate has been exceeded.

Therefore, the actual excess of soluble cupric ion over enzyme-bound FAD must be significantly less than 8-fold. However for consistancy of comparison of our results with earlier experiments in our own laboratory, and with the results of Casola et al. [6] who used substantially the same procedure for copper inactivation, the conditions of inactivation were not changed. Before and after the inactivation with cupric ion, the enzyme was assayed according to Casola et al. [6] for both lipoic acid reductase and diaphorase activities.

*Modification of the enzyme using concentrated iodoacetamide*

Native pig heart lipoamide dehydrogenase was inactivated by iodoacetamide according to the method suggested by Dr Abraham Stein (personal communication). The enzyme was incubated for 24 h, at 25 °C, in the dark, in a solution which was 0.1 M in both iodoacetamide and phosphate buffer, pH 7.2. At the completion of the reaction, the excess iodoacetamide was removed by passage over a Sephadex G-25 column. For studies of the effect of NAD on alkylation by concentrated iodoacetamide, a neutralized 0.5 M solution of NAD was prepared by addition of NaOH. Alkylation of the NAD-protected enzyme was performed in solutions which were 0.1 M in NAD, iodoacetamide and phosphate buffer, pH 7.2. Assays of the enzyme during the course of alkylation were performed by diluting a 10 $\mu$l aliquot of enzyme into 90 $\mu$l of 0.1 M cysteine (cysteine·HCl neutralized with NaOH to pH 7) and waiting 10 min for all iodoacetamide to react. Aliquots of enzyme–cysteine solution were then added to assay mixtures. Additions of comparable aliquots of 0.09 M cysteine to assay mixtures, containing native enzyme showed no influence on the measured activity in the NADH-lipoamide reductase assay. This technique could not be used to measure diaphorase activity since cysteine reacts rapidly with 2,6-dichloroindophenol in a non-enzymatic reaction.

## RESULTS AND DISCUSSION

*Modification with concentrated iodoacetamide*

Enzyme inactivated by alkylation in 0.1 M iodoacetamide shows 4–7% of its original lipoamide reductase activity in agreement with the activity reported by Stein and Stein [4] and approx. 60% of its original diaphorase activity. There is no apparent dissociation of flavin from the alkylated enzyme; on passage of the modified enzyme over Sephadex G-25, all the fluorescence and yellow color are associated with the protein fraction. Anaerobic reduction of the alkylated enzyme results in the apparently complete formation of the red 2-electron reduced enzyme, (the form seen as the intermediate in catalysis) with either NADH or reduced lipoamide as the reductant. This indicates that the residual 5% lipoamide reductase activity of alkylated enzyme is not due to 5% unmodified enzyme, but must be due to all of the enzyme turning over at a diminished rate.

The two halves of Fig. 1 compare the ability to form the NAD-dependent charge-transfer complex in enzyme alkylated in the absence or presence of NAD. The upper section of Fig. 1 shows our repetition of an experiment reported by Stein and Stein [4] in which enzyme previously alkylated with concentrated iodoacetamide is subjected to anaerobic reduction by an 8-fold excess of NADH over enzyme-bound FAD, in the presence of arsenite, leading to the production of the 4-electron reduced

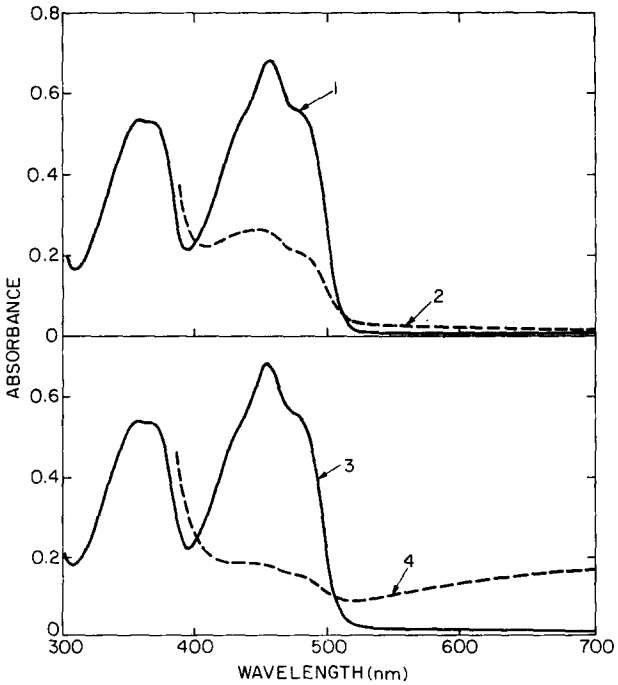


Fig. 1. (1) Spectrum of enzyme modified by alkylation in concentrated iodoacetamide as described in the text. Alkylation was allowed to proceed for 24 h and then the excess reagents were removed. The enzyme was dissolved in 0.1 M phosphate buffer, pH 7.6 containing 0.3 mM EDTA and 1 mM sodium arsenite. (2) Enzyme after anaerobic addition of 8 equivalents NADH per enzyme-bound FAD. (3) Spectrum of enzyme modified by alkylation by concentrated iodoacetamide in the presence of NAD as described in the text. The alkylation was allowed to proceed for 2 h and then the excess reagents were removed. The enzyme was dissolved in the buffer system described in (1). (4) Enzyme after anaerobic addition of 8 equivalents of NADH per enzyme-bound FAD.

enzyme. There is no long wavelength band formed upon reduction, although at least 2 equivalents of NAD have been produced. In comparison, in Fig. 1, lower section, we have shown that enzyme alkylated under the same conditions, but in the presence of 0.1 M NAD (later removed), retains its pyridine nucleotide binding site as evidenced by the formation of the green charge-transfer complex between NAD and $FADH_2$.

We have determined that alkylation of unprotected enzyme results in the carboxymethylation of two thiols and one methionine, and the time course of the alkylation is shown in Fig. 2. Comparison of the time course of alkylation in the presence and absence of NAD demonstrates that substantial protection against alkylation is afforded by the NAD. The spike in activity of the NAD protected enzyme during alkylation is not always observed. The inset shows the results of amino acid analysis of samples of enzyme taken at intervals during alkylation. It can be seen that methionine and one of the two thiols alkylate within 2 h in both unprotected and NAD-protected enzyme, and that NAD-protected enzyme is fully active at this stage in alkylation. Loss in activity appears to be associated with the alkylation of a second thiol which is substantially protected by NAD.

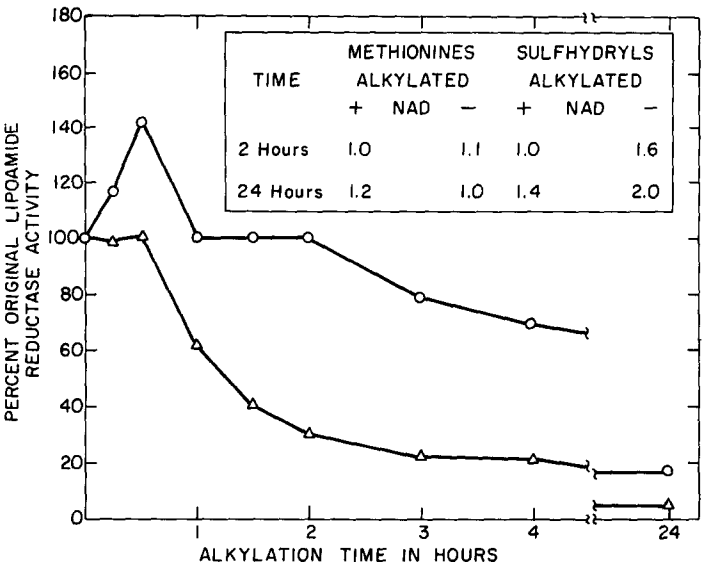


Fig. 2. The time course of alkylation of unprotected (△) and NAD-protected (○) heart lipoamide dehydrogenase. The alkylations and assays for activity of the enzyme were performed as described in the text. The inset summarizes the results of amino acid analyses performed on aliquots of enzyme solutions which were removed at intervals during the alkylation. A 5-fold excess of β-mercaptoethanol over iodoacetamide present in the aliquot was added to stop the reaction, and after 30 min the sample was prepared for hydrolysis.

If the unprotected enzyme is treated with unlabelled iodoacetamide for 24 h, so that two thiols and one methionine are alkylated, it can then be realkylated in the presence of dithiothreitol and guanidine, using iodo[$^{14}C_1$]acetate and digested with trypsin. Any thiol peptide alkylated with iodoacetamide should now be missing from the radioautogram of a two-dimensional chromatography–electrophoresis of the digest. Fig. 3 shows such a radioautogram. Three thiol peptides, Peptides 16, 18 and 51, normally seen in maps of reduced alkylated lipoamide dehydrogenase, are completely missing [7]. Identification of the labelled peptides is greatly assisted by staining the entire map with cadmium–ninhydrin, since the characteristic colors and grouping of the peptides are quite reproducible from one map to another, even though small differences in the chromatography or electrophoresis of a given peptide may occur.

If NAD-protected enzyme is alkylated for 2 h with unlabelled iodoacetamide, so that one thiol and one methionine are alkylated, the enzyme can be realkylated with iodo[$^{14}C_1$]acetate after incubation with dithiothreitol and guanidine, digested with trypsin, and peptide maps prepared. Radioautograms of enzyme prepared in this manner show no missing spots, but look identical to radioautograms of untreated enzyme, alkylated with iodo[$^{14}C_1$]acetate after reduction with dithiothreitol in guanidine.

Matthews et al. [7] demonstrated that Peptide 18 is derived from Peptide 51 by chymotryptic cleavage, and gave less conclusive evidence suggesting that Peptide 16 may be a related sequence. If Peptides 16 and 18–51 represent alternative sequences of a given thiol region, their absence demonstrates the alkylation of only one thiol group per enzyme-bound FAD. The radioactive label in Peptide 16 accounts for only 10%

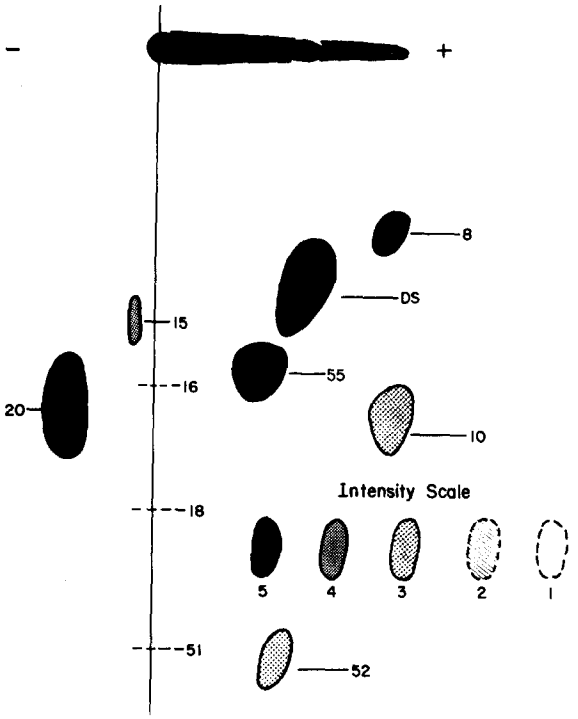


Fig. 3. A radioautogram of a peptide map of enzyme alkylated for 24 h with unlabelled concentrated iodoacetamide as described in the text, and then denatured, reduced and alkylated with iodo[$^{14}$C]-acetate, and digested with trypsin. The peptide map was prepared as described in the text. The origin is at the top on the vertical line. Descending chromatography was performed in the vertical dimension and pH 6.5 electrophoresis in the horizontal dimension. The cathode was to the left of the origin and the anode to the right. The numbers associated with dashed lines indicate the positions on the map where peptides, seen in maps of unmodified enzyme, are absent.

of one thiol per FAD, while the label in Peptides 18 and 51 accounts for 70% of one thiol.

The thiol which is rapidly alkylated by iodoacetamide is presumably located in one of the two reactive thiol regions which are not released by tryptic cleavage. These regions have been isolated by Brown and Perham [11] using tryptic–chymotryptic digestions and have been sequenced. The sequences (Peptides DTC1a and DTC1b3) are shown in Table IV of Matthews et al. [7]. The thiol which reacts rapidly with iodoacetamide is not unreactive to iodoacetate, since the same number of thiol groups (8.3) are alkylated whether enzyme is reduced and alkylated with iodoacetate, or first reacted with iodoacetamide and then reduced and alkylated with iodoacetate.

Thus the thiol in Peptide 16 and/or the thiol in Peptide 18–51 appears to be at or very near an NAD-binding site on the enzyme, and its alkylation leads to loss of this binding site.

The loss of the NAD-binding site is evidenced by abolition of the charge-transfer complex between $FADH_2$ and NAD. But NAD also protects oxidized enzyme against alkylation, presumably by binding so as to mask the thiol at or near the NAD-binding site which functions in reduced enzyme. Thus, we feel that oxidized

enzyme must also bind NAD at the same site as in reduced enzyme. Small spectral changes are indeed observed when NAD is added to oxidized enzyme [12] or to the red catalytic intermediate [13].

There is experimental evidence that oxidized enzyme contains two distinct pyridine nucleotide binding sites [2, 12, 14]. It is reasonable to suppose that one of these sites represents the catalytic binding site for NADH or NAD since in vitro the enzyme catalyzes a reversible reaction. The NAD-binding site lost on alkylation is not the catalytic binding site, because the alkylated enzyme is still capable of catalysis albeit at a slower rate. A question might be raised as to whether loss of the green charge-transfer complex necessarily reflects loss of a pyridine nucleotide binding site. The charge-transfer complex could be abolished because the bound pyridine nucleotide was no longer sufficiently close to the flavin due to a conformational change induced by alkylation of the thiol group. But in this case, if alkylated enzyme were still to bind pyridine nucleotide, one would not expect the presence of NAD to protect against the alkylation of the thiol.

It is tempting to equate the pyridine nucleotide binding site lost on alkylation with the NAD-binding site ("Y" site) proposed by Massey and Veeger [2] in their mechanism for lipoamide dehydrogenase. The "Y" site was proposed to explain the requirement for the presence of NAD in order to observe full NADH-lipoate reductase activity.

Recently Voetberg and Veeger [12] have presented data indicating that the role of NAD in the NADH-lipoate reaction is to lower the p$K$ of a group functioning in catalysis in its deprotonated form, and they suggest that this group may be a thiol. If this is the case, it is almost certainly the thiol which, when alkylated, prevents charge-transfer interaction between $FADH_2$ and NAD.

*Enzyme modified by cupric ion*

We have attempted to identify the thiols which are oxidized to form a disulfide bond when heart lipoamide dehydrogenase is inactivated by cupric ion. The thiol titer of native enzyme reacted with DTNB is 6.9–7.0, but after treatment with cupric ion the titer drops to 4.9–5.3. The treated enzyme shows NADH-lipoamide reductase activity which is 4–10% that of native enzyme, and diaphorase activity which is 10–15-fold higher than native enzyme. If the copper-treated enzyme is anaerobically reduced by NADH in the presence of 1 mM arsenite (cf. the experiment in Fig. 1) there is complete formation of the green charge-transfer complex, and the spectrum of the reduced enzyme is indistinguishable from that of native enzyme reduced in the same way [5]. This led us to believe that the thiols involved in the disulfide bond formed on copper inactivation must not be the same as the thiol responsible for inactivation during alkylation in concentrated iodoacetamide. We attempted to identify the thiols oxidized during treatment with cupric ion by performing the following paired experiment. Enzyme was inactivated with cupric ion and the cupric ion removed. Cupric ion acts catalytically to oxidize the thiols to a disulfide and can be readily removed by dialysis [5]. The enzyme was divided into two fractions. Fraction 1 was alkylated with iodo[$^{14}C_1$]acetate and then was reduced with dithiothreitol and realkylated with unlabelled iodoacetate. Fraction 2 was first alkylated with unlabelled iodoacetate and then reduced and realkylated with iodo[$^{14}C_1$]acetate. The alkylations were carried out in 7.5 M guanidine·HCl. Both fractions were digested with trypsin

and peptide maps prepared. In this manner thiols involved in the two disulfide bonds in enzyme treated with cupric ion should appear in radioautograms of Fraction 2 and be absent in Fraction 1 radioautograms. The results of a typical experiment are shown in Fig. 4; the radioautogram of the Fraction 1 map is on the left and that of the Fraction 2 map is on the right. The two peptides (Peptides 15 and DS) representing the active center disulfide [7] are prominent in Fraction 2 radioautograms, as expected. In addition, Peptides 20, 8 and 10 are more prominent in Fraction 2 radioautograms than in Fraction 1 radioautograms. The interpretation of the results of experiments like these are subject to an important caveat. It is clear from the appearance of some labelled Peptide DS in Fraction 1 radioautograms that some disulfide interchange is taking place in copper-treated enzyme. This stands in contrast to native enzyme where the same experiment leads to the almost total absence of Peptide DS from Fraction 1 radioautograms (Fig. 1, ref. 7). The conditions of efficient alkylation with iodoacetate, pH 8, 7.5 M guanidine, are obviously favorable for disulfide interchange and we have not been able to alkylate cupric ion-treated enzyme without some disulfide interchange. This may explain the fact that Peptides 8, 10 and 20 are

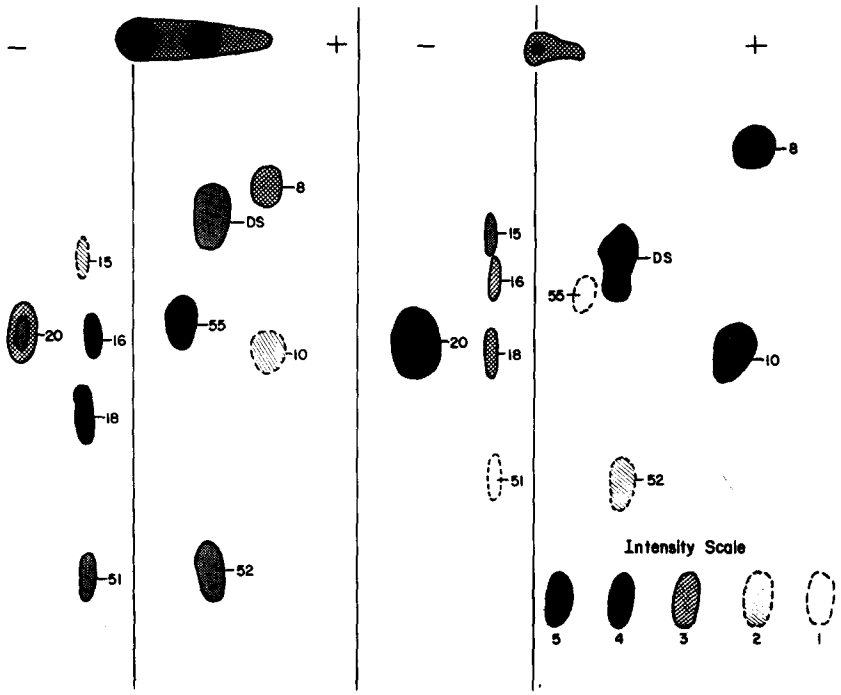


Fig. 4. On the left is a radioautogram of a peptide map of a tryptic digest of lipoamide dehydrogenase inactivated by cupric ion and alkylated as follows: the denatured enzyme is alkylated with iodo[$^{14}C_1$]-acetate and then reduced and realkylated with unlabelled iodoacetate. On the right is a radioautogram of a peptide map of a tryptic digest of enzyme inactivated by cupric ion which has been denatured and alkylated with unlabelled iodoacetate and then reduced and realkylated with iodo[$^{14}C_1$]acetate. The preparation of the peptide map is described in the text, and the orientation of the maps is the same as the map in Fig. 3. Identical amounts of digested protein, as determined by amino acid analysis, 25 nmoles, were used for the two peptide maps.

not completely absent from the Fraction 1 radioautograms. Matthews et al. [7] discuss the evidence indicating that Peptide 8 is derived from Peptide 10 by chymotryptic cleavage, secondary to the tryptic digestion. The relative amounts of Peptides 8 and 10 produced in any given tryptic digestion seem to be quite variable. Thus the disulfide bond formed by the catalytic action of cupric ion appears to involve a unique pair of thiols, the thiol in Peptide 20 and that in Peptide 10–8. This implies that these two thiols lie reasonably close to one another in the three-dimensional structure of the native enzyme. It is of interest to speculate that their oxidation to a disulfide induces a strain in the molecule which disrupts the close juxtaposition of the flavin and the redox active disulfide. This juxtaposition is essential for the stability of the 2-electron reduced form that is functional in the lipoate reaction. The transient formation of this intermediate in cupric ion treated enzyme occurs upon reduction [5] indicating that the movement induced in the protein by the formation of a new disulfide is small.

Dr Colin Thorpe, in our laboratory, has suggested that the histidines in Peptide 20 may serve to bind the cupric ion prior to metal catalyzed disulfide formation. Brown and Perham [11] have sequenced the amino-terminal portion of Peptide 20. Their sequence (Val–Cys(Cm)–His–Ala–His–Pro...) indicates that the histidines and this cysteine are indeed in close proximity to one another. There are several precedents for cupric ion binding to specific histidine residues in protein [15, 16]. Such an "anchoring" of the cupric ion to the protein, might help to explain the specificity and rapidity of the effect of cupric ion on a protein with so many thiol residues.

Chemical modification of certain thiols in pig heart lipoamide dehydrogenase leads to profound alterations in the catalytic activities of this enzyme. Although lipoate reductase is lowered in both modifications of the enzyme discussed here, diaphorase activity is lowered on alkylation of the enzyme and raised on treatment with cupric ion. The pyridine nucleotide binding site associated with charge transfer between NAD and $FADH_2$ is unaffected in the cupric ion treated enzyme while it is lost in enzyme reacted with iodoacetamide. The results reported here have established the identity of the cysteine residues involved in these modifications. The thiols oxidized to a disulfide by the catalytic action of cupric ion have been shown to be distinct from the thiols alkylated by iodoacetamide.

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REFERENCES

1 Massey, V., Gibson, Q. H. and Veeger, C. (1960) Biochem. J. 77, 341–351
2 Massey, V. and Veeger, C. (1961) Biochim. Biophys. Acta 48, 33–47

3 Massey, V. and Palmer, G. (1962) J. Biol. Chem. 237, 2347–2358
4 Stein, A. M. and Stein, J. H. (1971) J. Biol. Chem. 246, 670–676
5 Veeger, C. and Massey, V. (1962) Biochim. Biophys. Acta 64, 83–100
6 Casola, L., Brumby, P. E. and Massey, V. (1966) J. Biol. Chem. 241, 4977–4984
7 Matthews, R. G., Arscott, L. D. and Williams, Jr, C. H., (1974) Biochim. Biophys. Acta 370, 000–000
8 Matthews, R. G. and Williams, Jr, C. H. (1973) Fed. Proc. 32, Abstract No. 2503, 657
9 Gurd, F. R. N. (1967) Methods Enzymol. 11, 532–541
10 Massey, V. (1960) Biochim. Biophys. Acta 37, 314–322
11 Brown, J. P. and Perham, R. N. (1974) Biochem. J. 137, 505–512
12 Van Muiswinkel-Voetberg, H. and Veeger, C. (1973) Eur. J. Biochem. 33, 285–291
13 Veeger, C. and Massey, V. (1963) Biochim. Biophys. Acta 67, 679–681
14 Su, G. and Wilson, J. E. (1971) Arch. Biochem. Biophys. 143, 253–260
15 Peters, Jr, T. and Blumenstock, F. A. (1967) J. Biol. Chem. 242, 1574–1578
16 Forman, H. J., Evans, H. J., Hill, R. L. and Fridovich, I. (1973) Biochemistry 12, 823–827