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Modification of Pig Heart Lipoamide Dehydrogenase by Cupric Ions†

Colin Thorpe and Charles H. Williams, Jr.*

ABSTRACT: The insertion of a second disulfide bridge into native pig heart lipoamide dehydrogenase, requires two Cu^{2+} ions for each catalytic center inactivated under anaerobic conditions. During inactivation, both metal atoms become reducible by their juxtaposition to the two participating cysteine residues and may be removed as the Cu^{+}-chelates of neocuproine and bathocuproinesulfonate, leaving an additional disulfide bridge on the protein. Inactivation does not require the presence of oxygen, but when substoichiometric levels of copper are used under aerobic conditions the slow regeneration of Cu^{2+} becomes rate-limiting. The course of aerobic inactivation is markedly biphasic at 0° using 2 Cu^{2+}/FAD, with 30% of the total change completed rapidly, followed by a much slower phase. Both the extent of the fast phase and the rate of the second phase are enhanced by increasing levels of Cu^{2+}, but are relatively unaffected when the Cu^{2+}/FAD ratio is maintained at 2 and the protein concentration is varied. The enzyme affords several binding sites for Cu^{2+} at pH 7.8, and it is suggested that competition between these sites during the initial statistical distribution of metal ions may explain this biphasic behavior.

Pig heart lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3), a component of both the pyruvate and α-ketoglutarate dehydrogenase multienzyme complexes, catalyzes the following reaction (Massey, 1963):[1]

$$NAD^{+} + Lip(SH)_2 \rightleftharpoons NADH + Lip(S\text{–}S)$$

The enzyme, as isolated, is a dimer with one FAD (Massey et al., 1962), a single disulfide bond, and seven cysteine residues titratable with 5,5′-dithiobis(2-nitrobenzoic acid) per polypeptide (Matthews et al., 1974). The FAD and disulfide moieties comprise part of the active site and interact electronically during catalysis (Massey and Veeger, 1961). Veeger and Massey (1962) demonstrated that lipoamide dehydrogenase is readily inactivated by low levels of Cu^{2+} yielding a protein with a much lower activity in the NADH/Lip(S–S) assay but an enhanced DCI reductase activity (approximately 3 and 2000% that of the native protein, respectively). It was subsequently demonstrated that copper treatment introduces one or more additional disulfide bonds

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[1] Abbreviations used are: BCS, bathocuproinesulfonate; DCI, 2,6-dichloroindophenol; $Lip(SH)_2$ and Lip(S–S), reduced and oxidized lipoamide, respectively.

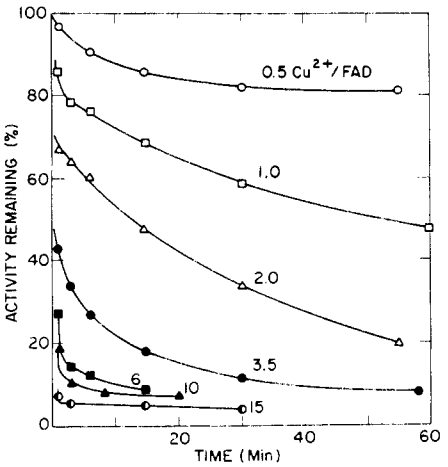


FIGURE 1: The inactivation of lipoamide dehydrogenase with Cu^{2+}. Enzyme in 50 m*M* Tris-Cl buffer (pH 7.9) was equilibrated at 0° in small tubes and 10 μl was withdrawn and diluted into 0.3 ml of quenching solution (20 m*M* EDTA in 100 m*M* phosphate buffer (pH 7.6) at 0°) to establish the initial activity of the native enzyme. The reaction was started by the addition of 20 μl of Cu^{2+} solution to give a total of 0.21 ml of buffer containing 21 μM enzyme and 10.5–315 μM Cu^{2+} as indicated. Further aliquots were withdrawn during the incubation and rapidly diluted in quenching solution as before. The results are expressed as percentages of the initial activity in the NADH/Lip(S-S) assay.

per FAD. Further, dialysis of the modified protein against cysteine regenerates the native enzyme provided that copper treatment is not prolonged (Casola et al., 1966). Work in this laboratory has confirmed these findings, with the isolation of peptides containing the two cysteine residues participating in the copper catalyzed disulfide bond formation (Matthews and Williams, 1974). One of these peptides contains two histidine residues and is that designated as DT2n2 by Brown and Perham (1974) in their study of the cysteine peptides of the enzyme. This peptide has the sequence: Val-Cys-His-Ala-His-Pro(Thr,Ser,Glx,Ala,Leu,Phe).

Proteins and peptides may afford binding sites of high affinity for Cu^{2+} at neutral pH via a combination of the imidazole ring of histidine residues and nitrogen atoms of adjacent peptide bonds (Bryce et al., 1965; Peters and Blumenstock, 1967). The specificity and facility with which disulfide formation occurs in lipoamide dehydrogenase might be explained, therefore, by Cu^{2+} first binding to this histidine-containing peptide and subsequently interacting with the adjacent cysteine residues. The mechanism by which copper catalyzes disulfide bond formation in proteins is imperfectly understood, although the reaction is known to occur in several systems. Lipoamide dehydrogenase from bakers' yeast (Wren and Massey, 1966) and from *Escherichia coli* (Williams, unpublished) are considerably less sensitive than the heart enzyme to inactivation by copper. Boothe and Folk (1969) have studied the copper-catalyzed inactivation of guinea pig liver transglutaminase. Here, complete loss of activity is accompanied by the formation of two intramolecular disulfide bridges. This inactivation is rapidly reversed with dithiothreitol. In a model system, Cavallini et al. (1969) have shown that the oxidation of cysteine by catalytic levels of copper in aerobic alkaline solution proceeds through the formation of a biscysteine-Cu^{2+} chelate. Reduction of Cu^{2+} to Cu^{+} occurs via a rate-limiting redox dissociation of one coordinated sulfur atom as a thiyl radical (Blumberg and Peisach, cited in Cavallini et al., (1969). However Cu^{+} was not detected during catalysis by either physical or chemical methods, since its reoxidation by O_2 occurs much faster then its rate of production. The resulting thiyl radical dissociates from the complex to be replaced by another cysteine molecule. Cystine is formed via the dimerization of two such radicals.

We have reinvestigated the copper-catalyzed inactivation of pig heart lipoamide dehydrogenase and suggest a mechanism for this process.

Materials and Methods

Pig heart lipoamide dehydrogenase was obtained from either Boehringer or Sigma and was further purified using a calcium phosphate gel-cellulose column as described previously (Williams et al., 1967). The enzyme is routinely purified and stored in 0.1 *M* phosphate buffer (pH 7.6) containing 0.3 m*M* EDTA. For these experiments, the protein was equilibrated with 50 m*M* Tris-Cl buffer (pH 7.8) at 4°, either by gel filtration using a Sephadex G-25 column or by dialysis. Concentrations of enzyme are expressed with respect to flavine using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm (Massey et al., 1962). NADH/Lip(S-S) assays of enzyme activity were performed at pH 6.3 as described previously (Thorpe and Williams, 1974).

Copper Determinations. Stock solutions of $CuSO_4$ (Baker Analyzed) were prepared in the appropriate buffers. Copper was routinely determined in the presence of 0.9–1.0 m*M* neocuproine (10 m*M* in 50% v/v methanol) after the addition of one crystal of ascorbic acid. The color of the Cu^{+}–neocuproine complex develops rapidly and is stable. An extinction coefficient of 7.28 mM^{-1} cm^{-1} at 450 nm was determined for this complex in 50 m*M* Tris-Cl. For the analogous BCS complex a value of 12.1 mM^{-1} cm^{-1} at 480 nm was used in good agreement with that quoted by Diehl and Smith (1958). BCS and neocuproine were obtained from G. F. Smith Chemical Co.

Anaerobic Incubations. Anaerobic incubations of the enzyme were conducted in a small test tube (10 × 46 mm internal dimensions) sealed with a rubber stopper pierced by two pieces of 20 gauge stainless steel hypodermic tubing. This system allowed a slow stream of nitrogen (Linde, ultra high purity) to be passed over the liquid surface. A Hamilton syringe needle, fitting closely through the gas exit tube, was used to withdraw samples from the incubation. The contents of the tube were initially deoxygenated by placing the tube and attached stopper into a larger vessel which could be evacuated and flushed with nitrogen.

Other Methods. Visible spectra were recorded on a Cary 118 C spectrophotometer. Dry air was forced through the cell compartment to avoid condensation when the cuvets were maintained at 5°. Assays were conducted using a Beckman DU instrument.

Results

Figure 1 shows the inactivation of pig heart lipoamide dehydrogenase in 50 m*M* Tris-Cl (pH 7.9) at 0° with various levels of copper. The course of thiol oxidation was followed by mixing aliquots of the incubation medium with a large molar excess of EDTA. This procedure would be expected to quench the reaction efficiently since the stability constant for the Cu^{2+} chelate is approximately 10^{17} (Sillén and Martell, 1964). Partially inactivated enzyme quenched in this manner showed no significant change in activity over several hours at 0°. The enzyme is rapidly inactivated when the Cu^{2+}/FAD ratio is greater than 6 (Figure 1). At lower concentrations of copper the inactivation is clearly biphasic,

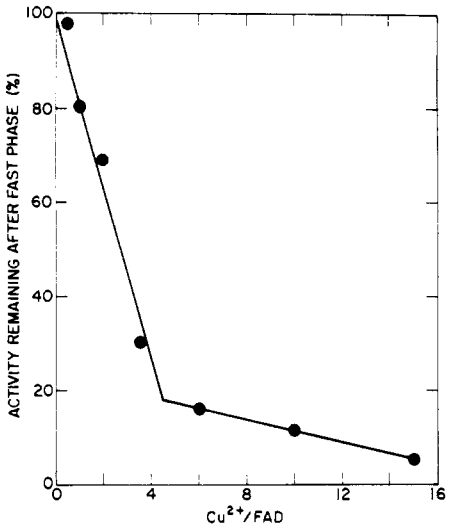


FIGURE 2: The percentage activity remaining after the fast phase as a function of added copper. The activity remaining after the fast phase was obtained as described in the text.

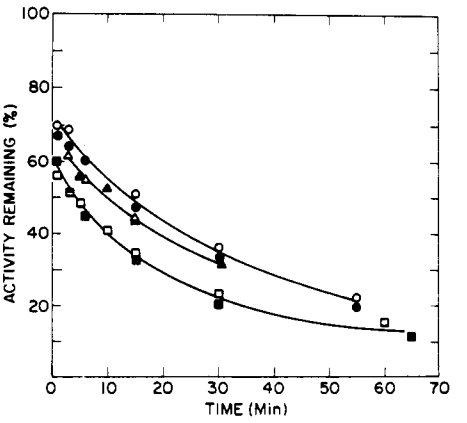


FIGURE 3: The effect of protein concentration on the inactivation of lipoamide dehydrogenase at a Cu^{2+}/FAD ratio of 2.0. The experiment was performed as described in the legend to Figure 1 except that the protein concentrations were: 10.9 μM, (triangles); 21 μM, (circles); and 36 μM, (squares); and the Cu^{2+}/FAD ratio was 2.0. The open and solid symbols represent duplicate experiments.

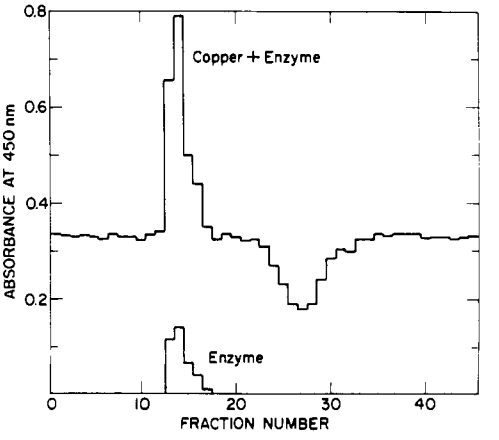


FIGURE 4: Binding of copper to lipoamide dehydrogenase by the gel filtration method. A Sephadex G-25 column (29 × 0.9 cm) was equilibrated at 4° with 50 mM Tris-Cl (pH 7.8) containing 50 μM Cu^{2+}. Enzyme was mixed with copper to give 26 nmol of FAD in 0.253 ml of buffer of the same composition and immediately applied to the column. Fractions of 0.66 ml were collected at a flow rate of 6.3 ml/hr. The absorbance at 450 nm was determined using 0.6-ml aliquots, remeasured after the addition of 60 μl of 10 mM neocuproine (in 50% v/v methanol) and again after the addition of one crystal of ascorbic acid. This allowed the levels of FAD, reduced copper, and total copper in each fraction to be determined. The top profile is the total absorption due to both flavine and copper, the lower profile the flavine contribution alone.

with a rapid loss followed by a slower decline in activity. The rate of this second phase is enhanced by increasing levels of copper. No loss of activity is observed in the absence of added copper over 1 hr at 0°. An estimation of the extent of the rapid phase for each metal concentration has been made by replotting the results of Figure 1 on semilogarithmic paper and extrapolating the linear portions of the slow phases to zero time. Figure 2 shows these extrapolated values as a function of Cu^{2+} concentration. The points fall roughly on two straight lines which intersect at a point corresponding to 4–5 Cu^{2+}/FAD.

Figure 3 shows the inactivation of the enzyme at a fixed Cu^{2+}/FAD ratio of 2 at three protein concentrations. The curve corresponding to 10.9 μM enzyme lies between those of 21 and 36 μM. The percent activity remaining after the fast phase and the rates of the subsequent slow phases do not differ widely between the three concentrations.

One possible explanation for the results shown in Figures 1 and 2 is that metal binding sites on the protein afford some protection to the native enzyme, by lowering the concentration of free Cu^{2+}. Binding of Cu^{2+} to lipoamide dehydrogenase was examined by the method described by Hummel and Dreyer (1962). The enzyme was applied to a column of G-25 Sephadex in buffer containing 50 μM Cu^{2+}. The equilibration of the column and the subsequent elution of the protein were performed with the same buffer. Copper was determined as the yellow Cu^{+}–neocuproine complex after reduction of the Cu^{2+} with ascorbate (see Materials and Methods). The flavoprotein elutes in fractions 13–17 (Figure 4), and carries with it an excess of metal ions over the equilibrium level. The area of the resulting trough corresponds to 3.1 copper atoms bound/FAD under these conditions. Some of the copper eluting with the protein was in the reduced form, with the addition of neocuproine alone giving a rapid appearance of yellow color equivalent to approximately 0.7 Cu^{+}/FAD. A control experiment, in which Cu^{2+} was added to a mixture of the native protein and neocuproine, showed that the unmodified enzyme does not reduce Cu^{2+} in the presence of this reagent. An attempt was therefore made to correlate the extent of inactivation of the enzyme with the production of Cu^{+} under aerobic conditions.

Lipoamide dehydrogenase was incubated at 0° with 2.5 Cu^{2+}/FAD and samples were withdrawn for Cu^{+} determinations and for activity measurements (Figure 5). As the inactivation proceeds, the concentration of Cu^{+} detected reaches a maximum corresponding to approximately 1.3 Cu^{+}/FAD and then declines. Expressing the results in terms of the Cu^{+} produced for each catalytic center inactivated the ratios become 2.1, 1.7, 1.3, and 0.9 Cu^{+}/FAD inactivated after 1, 9, 28, and 60 min of incubation, respectively. Figure 5 also shows results of a comparable incubation under anaerobic conditions (see Materials and Methods). It is evident that there is little difference between the rate of inactivation in the presence or absence of oxygen. However, anaerobically a value of 1.9 Cu^{+} detected/FAD inactivated is maintained throughout the incubation instead of falling slowly as in the aerobic case (Figure 5). Similar results were obtained using BCS which, unlike neocuproine, is highly water soluble. The enzyme, which had been incu-

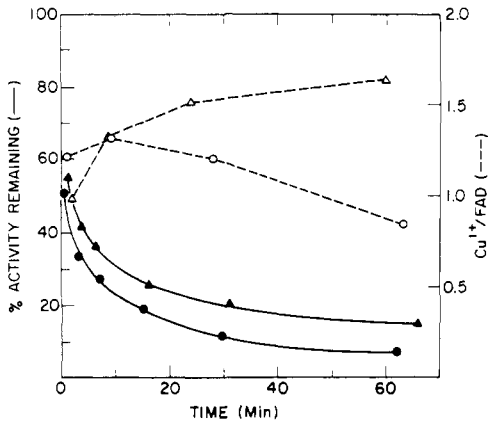


FIGURE 5: The effect of oxygen on the inactivation of lipoamide dehydrogenase and on the production of Cu^+ detectable using neocuproine. Enzyme was equilibrated at 0° under either aerobic (circles) or anaerobic (triangles; see Materials and Methods) conditions, and 11.9 μl of 5 mM Cu^{2+} solution was added to give 0.612 ml of Tris-Cl buffer (pH 7.9) containing 39 μM enzyme and 97.5 μM Cu^{2+}. Activities (●, ▲) were measured by using 7-μl aliquots rapidly diluted into 0.3 ml of quenching solution at 0°. Separate samples of 0.1 ml were mixed with 10 μl of 10 mM neocuproine (in 50% v/v methanol) at 0° and rapidly transferred to microcells. The increase in absorbance at 450 nm, recorded after subtracting the contribution of enzyme bound flavine at this wavelength, yields the Cu^+ detected/total FAD ratio shown (○, △).

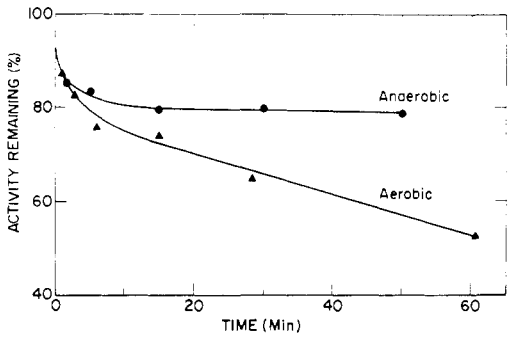


FIGURE 6: The effect of oxygen on the inactivation of lipoamide dehydrogenase using 0.5 Cu^{2+}/FAD. Enzyme was equilibrated at 22° in Tris-Cl (pH 7.2) under either aerobic (▲) or anaerobic (●) conditions, and a 10-μl aliquot was withdrawn for measurement of initial activity as described previously. The reaction was started by the addition of copper to give 17 μM Cu^{2+} and 33.9 μM enzyme in a total volume of 0.42 ml.

bated anaerobically with 1.5 Cu^{2+}/FAD until the activity had fallen to 34% of the native value, was mixed with 1.66 mM BCS. The resulting rapid increase in absorbance at 480 nm corresponded to two molecules of the Cu^+-BCS chelate/catalytic center inactivated. Separation of the protein from the metal-chelate and excess reagent was readily achieved using a Sephadex G-25 column. After gel filtration, the enzyme showed the expected optical spectrum with no evidence of bound BCS or of a mixed BCS-enzyme-copper species.

The stoichiometry of two Cu^{2+} required for each catalytic center inactivated is further supported by the results shown in Figure 6. Here, enzyme was incubated at 22° in Tris-Cl buffer (pH 7.2) with 0.5 Cu^{2+}/FAD. Anaerobically, this level of copper inactivates 22% of the protein—a value close to the expected 25%. Aerobically, regeneration of Cu^{2+} allows the reaction to proceed beyond this stoichiometry, as shown by the slower phase in Figure 6. The higher temperatures used in this experiment enhanced the rate

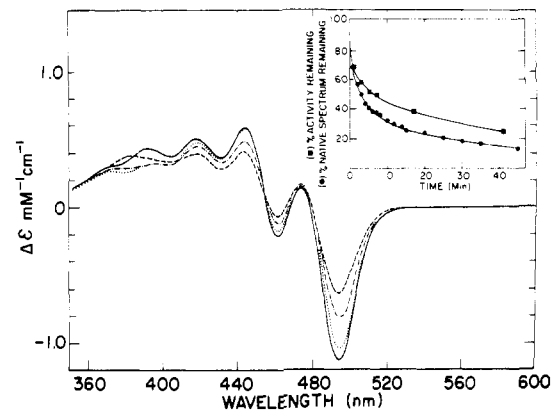


FIGURE 7: Difference spectra, copper modified-native spectrum, and the concomitant change in NADH/Lip(S-S) activity during inactivation at 5°. Two cuvets (1 ml volume, 10-mm path length) containing 0.59 ml of 36.1 μM enzyme in 50 mM Tris-Cl (pH 7.8) were equilibrated at 5° and a base line was recorded. The reaction was started by the addition of 6.4 μl of 5 mM Cu^{2+} in Tris-Cl to the sample cuvet (1.5 Cu^{2+}/FAD) after the addition of an equal volume of buffer alone to the reference cell. Spectra were recorded at 0.5 nm/sec at 0.5 min (- - - -); 12.5 (- · · · · · -); 147 (· · · ·); and 407 min(—) after mixing. The inset shows the result of an identical experiment following the reaction both by the change in absorbance at 495 nm (●) and by the loss in activity (■).

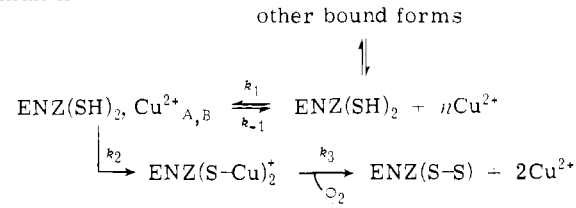
of the fast phase (compare with 0.5 Cu^{2+}/FAD in Figure 1) allowing a sharper delineation between the two phases.

The visible spectrum of copper-treated enzyme, freed of metal ions by dialysis, shows slight, but distinct, differences from that of the native enzyme. On treatment with copper, the absorption peak centered at 455 nm shifts approximately 3 nm to the blue, the shoulder at 480 nm becomes less pronounced, and the near-ultraviolet band is slightly intensified (Casola, et al., 1966). Figure 7 shows the reaction between Cu^{2+} and the enzyme followed by difference spectroscopy with protein plus 1.5 Cu^{2+}/FAD in the front cuvet. Spectroscopically distinct intermediates are not evident between the native and the copper-modified enzymes. The inset shows the course of the reaction monitored both by the decrease in absorbance at 495 nm and by the loss in activity. It can be seen that although these two parameters show the same general behavior the extent of spectral change exceeds the percentage of inactivation by approximately 10% in the slower phase of the reaction.

Discussion

The following model is proposed to account for the inactivation of lipoamide dehydrogenase by Cu^{2+}. The enzyme affords 4–5 binding sites for Cu^{2+} of appreciable affinity at pH 8.0; two of these sites (A and B) are at, or adjacent to, the cysteine residues participating in disulfide bond formation (Scheme I). The initial statistical distribution of metal

Scheme II



ions between the various sites on one subunit and between subunits themselves will depend on the various k_{on} constants appropriate to each binding site. Thus on mixing the

enzyme with 3 Cu^{2+}/FAD, only a certain fraction of the protein molecules carry two cupric ions closely juxtaposed to the two cysteine residues. This fraction is then rapidly inactivated via interaction between Cu^{2+} and thiol moieties and this process corresponds to the fast phase seen in Figure 1 ($k_2 \gg k_{-1}$). The slow phase of inactivation is then the rate at which Cu^{2+} atoms dissociate from binding sites in the protein population to reassociate with sites A and B on the remaining molecules of native enzyme.

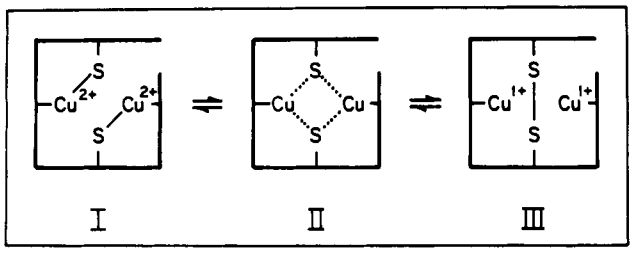
The formation of a disulfide bond from two cysteine residues is a two-electron oxidation and would therefore be expected to require two Cu^{2+} ions in the absence of alternative oxidants. The results of Casola et al. (1966) and those shown in Figure 6 confirm this stoichiometry. During inactivation this copper becomes detectable as the Cu^{+}-neocuproine complex on the addition of this chelating agent. The possibility that Cu^{+} exists free in solution in appreciable concentration is unlikely. Many cuprous species are rapidly oxidized by oxygen in aqueous solution whereas the Cu^{+} detectable by neocuproine decreases rather slowly in the aerobic experiment described in Figure 5. Cuprous copper is also subject to the disproportionation reaction (Cotton and Wilkinson, 1972):

$$2Cu^{+} \longrightarrow Cu^{2+} + Cu^{0}$$

Further, approximately 0.7 Cu^{+}/FAD was eluted with the enzyme during the metal binding experiment (Figure 4). Since the column itself was equilibrated with Cu^{2+}, the enzyme would continually have encountered a free metal concentration of 50 μM. Complete inactivation would have occurred within the first few centimeters of gel thus permitting a resolution of protein from any free Cu^{+} so generated.

Caution must be used in the interpretation of results obtained using "valence specific" reagents since they do not necessarily excise the metal in the oxidation state it occupies in the protein (Malmström, 1965). It is reasonable to assume, however, that the cupric atoms designated A and B in Scheme I become reducible during inactivation by their juxtaposition to the two thiol residues. Scheme II illustrates

Scheme I



a plausible mechanism by which such interactions could occur. Model studies reported by Hemmerich (1966) using low molecular weight thiols in an acetonitrile solvent system show that such equilibria are feasible. The actual state of the copper and sulfur moieties in the inactivated enzyme (represented by ENZ(S-Cu)$_2^{+}$ in Scheme I) is unknown. Indeed, if interconversions of the type shown in Scheme II occur and are very rapid, the valence of copper may be indeterminable (Hemmerich, 1966). The observation that the spectroscopic changes accompanying inactivation (Figure 7) are those expected for the production of copper-free diaphorase (where the additional disulfide is unequivocally present) is not conclusive evidence that structure III is the predominant form. Thus both forms II and III may involve a similar spatial relationship between the two cysteine containing peptides, and therefore exert a similar modification of the FAD environment and visible spectrum.

The slow disappearance of Cu^{+} detectable by neocuproine during the aerobic inactivation of the enzyme by 2.5 Cu^{2+}/FAD suggests that oxygen does not interact rapidly with ENZ(S-Cu)$_2^{+}$ ($k_3 < k_2$, Scheme I). This is supported by the slowness of the second phase of inactivation of the protein by 0.5 Cu^{2+}/FAD under aerobic conditions (Figure 6). Thus differences in the rate of inactivation between aerobic and anaerobic conditions are only marked when substoichiometric, catalytic, levels of copper are used, where the regeneration of free Cu^{2+} becomes rate-limiting. It should be noted that the mechanism of the reaction of ENZ(S-Cu)$_2^{+}$ with oxygen is unknown.

It might be expected that the addition of EDTA, a chelator exhibiting a thermodynamic preference for Cu^{2+} ($K_{Cu^{+}}$ approximately 10^7; Bridgart and Wilson, 1973) would reverse the inactivation by removing the Cu^{2+} from structure I in Scheme II. This is not the case. Partially inactivated enzyme quenched in aerobic EDTA solution showed no change in activity over several hours. Similarly, the addition of deoxygenated EDTA solution to 14 mM in the anaerobic experiment shown in Figure 5 at 68 min failed to reactivate the enzyme significantly after a further 2-hr incubation. It is possible that copper is initially excised from ENZ(S-Cu)$_2^{+}$ by EDTA as the Cu^{+}-chelate leaving an intact disulfide bond, in analogy with the behavior of BCS and neocuproine.

Although an attractive hypothesis, there is as yet no firm evidence that Cu^{2+} binds to histidine residues prior to the catalysis of disulfide bond formation. However, we suggest that those proteins which undergo rapid and specific oxidation by Cu^{2+} must both provide a suitable coordination environment for the metal, and also permit the close approach of the two participating cysteine residues to this site.

Acknowledgments

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Partial Purification and Properties of an Enzyme from *Escherichia coli* that Catalyzes the Conversion of Glutamic Acid and 10-Formyltetrahydropteroylglutamic Acid to 10-Formyltetrahydropteroyl-γ-glutamylglutamic Acid†

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ABSTRACT: An enzyme that catalyzes the conversion of L-glutamic acid and 10-formyl-H_4folic acid (also known as 10-formyl-H_4pteroylglutamic acid) to 10-formyl-H_4pteroyl-γ-glutamylglutamic acid has been purified by 74-fold from extracts of *Escherichia coli*. ATP, Mg^{2+}, and a monovalent cation (K^+ or NH_4, but not Na^+) are required for the enzyme to function. Radioactive and bioautographic analyses revealed the formation of a single product. This product was identified as 10-formyl-H_4pteroyl-γ-glutamylglutamic acid from its spectral characteristics, its ability to be used effectively as a growth factor for *Lactobacillus casei* 7469, and from radioactive analysis that indicated the incorporation into the product of 1 mol of glutamate/mol of 10-formyl-H_4pteroylglutamic acid utilized. The enzyme functions optimally at pH 9.0–9.8 and at 50°. Its molecular weight is estimated at 42,000–43,000. The K_m values are 180 μM for L-glutamic acid and less than 2 μM for (−)10-formyl-H_4pteroylglutamic acid. The only other naturally occurring folate compounds with significant activity as substrate are H_4pteroylglutamic acid and 5,10-methylene-H_4pteroylglutamic acid; however, these compounds are not used as effectively (K_m values are 10–12 μM) as 10-formyl-H_4pteroylglutamic acid.

Although it has been recognized for several years that nearly all of the naturally occurring forms of folate[1] are pteroylpolyglutamates (Pfiffner et al., 1946; Rabinowitz and Himes, 1960; Kozloff and Lute, 1965; Clandinin and Cossins, 1972; Houlihan and Scott, 1972), with the number of glutamate residues ranging from two (Usdin, 1959; Roos and Cossins, 1971; Shin et al., 1972) up to 12 (Kozloff and Lute, 1973), depending on the tissue analyzed, very little work has been done on the enzymology of the formation of these polyglutamate compounds. Several years ago, Griffin and Brown (1964) reported that H_4PteGlu could be converted in small quantities to the corresponding diglutamate in the presence of glutamic acid, ATP, and extracts of *Escherichia coli*. In the present paper, we report on our further investigations of this enzymatic process. Since the results to be presented indicate that the probable physiological substrate for the enzyme under investigation is 10-formyl-H_4PteGlu, we have here named the enzyme 10-formyltetrahydropteroyldiglutamic acid synthetase, or 10-formyl-H_4PteGlu$_2$ synthetase, for short.

Materials and Methods

Pteroylglutamic acid, *p*-aminobenzoic acid, dithiothreitol, all nonradioactive amino acids and nucleotides, DEAE-Sephadex A-50, Sephadex G-150, DNase I, RNase A, rabbit muscle aldolase, ovalbumin, and L-γ-glutamyl-L-glutamic acid were purchased from Sigma Chemical Co.;

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[1] In accord with the nomenclature rules recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, the following terminology is used: "folate" as the general term for the family of compounds; PteGlu, pteroylglutamic acid (folic acid); H_2PteGlu and H_4PteGlu, 7,8-dihydropteroylglutamic acid and 5,6,7,8-tetrahydropteroylglutamic acid, respectively; PteGlu$_2$, pteroyl-γ-glutamylglutamic acid; 10-formyl-H_4PteGlu, 10-formyltetrahydropteroylglutamic acid; 10-formyl-H_4PteGlu$_2$, 10-formyltetrahydropteroyl-γ-glutamylglutamic acid; 5,10-methylene-H_4PteGlu, 5,10-methylenetetrahydropteroylglutamic acid; 5,10-methenyl-H_4PteGlu, 5,10-methylidynetetrahydropteroylglutamic acid.