Identification of a Segment Containing a Reactive Cysteine Residue in Human Liver Cytoplasmic Aldehyde Dehydrogenase (Isoenzyme E_1)†

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ABSTRACT: A single cysteine residue is selectively alkylated by iodoacetamide in cytoplasmic human liver aldehyde dehydrogenase (isoenzyme E_1). The amino acid sequence of a 35-residue fragment containing this residue is determined, showing two additional cysteine residues and also three histidine residues. The alkylation is selective for Cys-30 of this fragment, with only little alkylation even at an adjacent residue, Cys-29. The region examined is likely to be of significance in the reaction of this isoenzyme with disulfiram since disulfiram blocks the selective alkylation.

Aldehyde dehydrogenases purified from mammalian liver are tetramers with subunits of about 500 residues. Two major isoenzymes have been identified: a cytoplasmic form with high sensitivity to the alcohol aversive agent disulfiram and a mitochondrial form less sensitive to this compound (Kitson, 1975; Eckfeldt & Yonetani, 1976a; Eckfeldt et al., 1976; Greenfield & Pietruszko, 1977; MacGibbon et al., 1979; Kitabatake et al., 1981). It is possible in man that both forms function in the metabolism of acetaldehyde derived from ingested ethanol (Kitson, 1977; Harada et al., 1980) although the relative importance of the two isoenzymes is not established.

In horse and man, the cytoplasmic isoenzyme is more basic than the mitochondrial isoenzyme. The two isoenzymes have extensive similarities in amino acid composition (Eckfeldt et al., 1976; Greenfield & Pietruszko, 1977), but substitutions are apparent as deduced from differences in peptide maps (Hempel et al., 1982; von Bahr-Lindström et al., 1982a). Both isoenzymes are thiol rich, containing eight to nine sulfhydryls per subunit (Eckfeldt et al., 1976; Hempel et al., 1982). A catalytic thiol in aldehyde dehydrogenase was proposed earlier (Racker, 1955), and one thiol has also been suggested to be close to the coenzyme-binding site (von Bahr-Lindström et al., 1981). However, direct labeling of a catalytic cysteine residue has yet to be demonstrated [cf. Weiner (1979)]. Despite the presence of many thiol groups per subunit, iodoacetamide reacts in an apparently selective manner with each of the major isoenzymes of human liver aldehyde dehydrogenase (Hempel & Pietruszko, 1981). This reaction is coincident with enzyme inactivation, facilitated by NAD, and prevented by aldehyde or the competitive inhibitor chloral. However, complete inactivation with iodoacetamide is not observed; the cytoplasmic (E_1) isoenzyme retains 5–10% and the mitochondrial (E_2) about 25% activity on reaction with this reagent.

In the present work, the residue reacting with iodoacetamide is identified and shown to be a single cysteine, the sequence around which is characterized. Furthermore, the significance of the iodoacetamide-reactive cysteine residue is enhanced by the finding that disulfiram blocks the selective alkylation. This cysteine differs from the one identified at the coenzyme-binding site of horse aldehyde dehydrogenase (von Bahr-Lindström et al., 1981), thus establishing the presence of another cysteine-containing region influencing catalytic activity of aldehyde dehydrogenases.

Materials and Methods

Protein. Human liver aldehyde dehydrogenase isoenzyme E_1 was isolated as described (Hempel et al., 1982). The enzyme used for selective alkylation by iodo[1-^{14}C]acetamide (13.1 mCi/mmol, New England Nuclear) had a specific activity of 0.35 unit/mg in the assay system used (Feldman & Weiner, 1972). Labeling was performed by incubation of E_1 (2 mg/mL) in 30 mM sodium phosphate (pH 7.0)–1 mM EDTA–0.7 mM NAD with iodo[^{14}C]acetamide. After 6.5 h, 25% residual enzyme activity was present, in agreement with previous results (Hempel & Pietruszko, 1981). The incubation was terminated by addition of mercaptoethanol, and after dialysis 1.3 equiv of carboxamidomethyl groups was found to be incorporated.

After lyophilization, the remaining protein SH groups were reduced with mercaptoethanol and carboxamidomethylated with unlabeled iodoacetamide, in 6 M guanidine hydrochloride –0.1 M Tris–2 mM EDTA, pH 8.0, as described (Hempel & Pietruszko, 1981). The reaction was stopped by addition of 0.2 volume of mercaptoethanol, and the protein was recovered by lyophilization after extensive dialysis against 1 mM HCl to remove salt. For the preparative isolation of the labeled peptide, the material was combined with an excess of unlabeled, carboxamidomethylated E_1 isoenzyme.

Analysis. Digestion of carboxamidomethyl-E_1 (2 mg/mL in 0.1 M ammonium bicarbonate) with TPCK–trypsin (Worthington) was performed with an enzyme to protein ratio of 1:50–1:100 at 34 °C for 24 h. After lyophilization, the peptide mixture was dissolved in 0.1 M formic acid and applied to Sephadex G-50 superfine, 1.6 × 90 cm (Pharmacia), equilibrated in the same solvent. Effluent was monitored at 226 nm by using a Varian 635 spectrophotometer. It was also screened by chromatography on thin-layer cellulose plates (Merck) in butanol–acetic acid–water–pyridine (15:3:12:10 by volume). Labeled fractions were pooled, lyophilized, dissolved in 20 mM acetic acid–6 M urea (Schwarz/Mann ultrapure), and applied to CM-52-cellulose (2.2 × 35 cm; Whatman) equilibrated in 20 mM sodium acetate, pH 3.65. The column was developed with 20 mM acetic acid–2 M urea and a linear gradient (0–140 mM) of NaCl. The major peak

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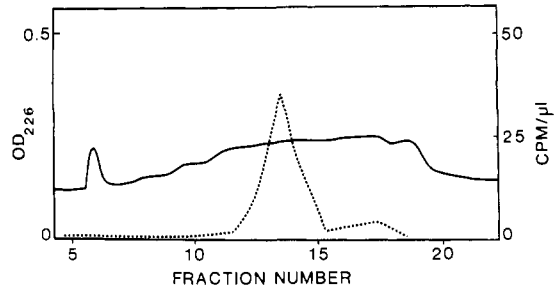

FIGURE 1: Sephadex G-50 chromatography in 0.1 M formic acid (fractions of 10.3 mL) of tryptic peptides from human liver aldehyde dehydrogenase E_1 (49 mg) selectively alkylated (1.3 equiv/tetramer) with iodo[^{14}C]acetamide. Solid line: absorbance at 226nm. Dotted line: radioactivity.

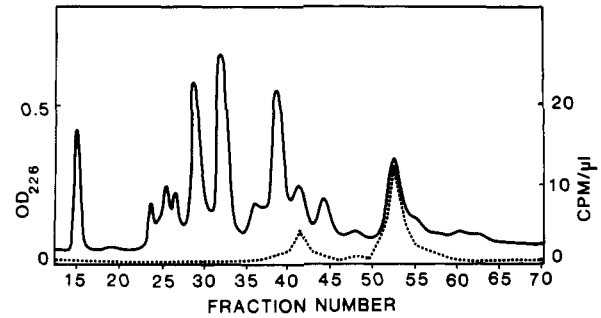

FIGURE 2: Chromatography on CM-52-cellulose of the labeled material from previous gel filtration (fractions 12–14 in Figure 1) of tryptic peptides of [^{14}C]carboxamidomethylated aldehyde dehydrogenase. The column was developed with a linear gradient of 0–140 mM NaCl in 2 M urea–0.02 M sodium acetate, pH 3.65. Solid line: absorbance at 226nm. Dotted line: radioactivity.

of unlabeled material was dialyzed (Spectrapor 3 dialysis tubing, Spectrum Medical Industries) against 2 mM HCl to remove urea and salt.

Manual sequence analysis was performed by the dansyl–Edman method using polyamide thin-layer chromatography for identification (Jörnvall, 1970) and by the DABITC method as described (Chang & Creaser, 1976; Chang et al., 1978), utilizing byproducts to assist the identification (von Bahr-Lindström et al., 1982b). Liquid-phase sequencer degradations were performed in a Beckmann 890C sequencer, as well as in one equipped with a cold trap, new valves, and a conversion cell as described (Wittmann-Liebold, 1980). Precycled polybrene (4 mg) and a 0.1 M Quadrol peptide program were used for degradations (Jörnvall & Philipson, 1980).

Redigestions with TLCK–chymotrypsin (Worthington) and with staphylococcal Glu-specific protease (Miles Laboratories) were performed in 0.1–0.2 M ammonium bicarbonate for 2–3 h at 37 °C at an enzyme:substrate weight ratio of 1:10–1:100. Chymotryptic peptides were isolated by high-performance liquid chromatography on an RP-18 column by using a semistepwise gradient of 0–60% propanol in 1 M pyridine–0.5 M acetic acid. An effluent shunt valve allowed diversion of a small portion of the effluent to a reaction cell for fluorometric detection of the peptides after reaction with fluorescamine (Roche). The fragments from redigestion with staphylococcal protease were similarly purified except for use of a gradient of 0–100% methanol in 0.1% trifluoroacetic acid for direct monitoring of the effluent at 215 nm.

Disulfiram Protection against Carboxamidomethylation. E_1 (1.2 mg/mL) was mixed with 4 equiv of disulfiram/tetramer, in thiol-free 30 mM sodium phosphate (pH 6.0)–1 mM EDTA. After dialysis against two changes of thiol-free 30 mM sodium phosphate–1 mM EDTA, pH 7.0, NAD was added to 0.7 mM and iodo[^{14}C]acetamide to 0.022 mM, providing 4 equiv/tetramer. Control E_1, which had not been subjected to disulfiram, was treated similarly. After 6.5 h, the incorporation of iodo[^{14}C]acetamide was determined. The disulfiram-pretreated and control enzymes were then separately subjected to CNBr cleavage and subsequent NaDodSO$_4$–polyacrylamide gel electrophoresis.

Results

Isolation of Fragment with Specifically Labeled Cysteine Residue. E_1 was specifically labeled with iodo[^{14}C]acetamide as described (Hempel & Pietruszko, 1981), yielding an incorporation of 1.3 groups/tetramer at a 75% inhibition of the enzymatic activity. This material was digested with TPCK–trypsin and submitted to exclusion chromatography on Sephadex G-50 superfine in 0.1 M formic acid. The elution profile reveals essentially one labeled peak, as shown in Figure 1. Autoradiography of aliquots from the fractions after separation on thin-layer cellulose plates demonstrates the presence of one major labeled peptide in fractions 12–14 and a minor labeled peptide in fraction 17. Fractions 12–14 constitute 80% of the label, while fraction 17 contains less than 10% of the total label applied to the column.

Table I: Total Compositions of the Labeled Tryptic Fragment (T2S) and of Secondary Chymotryptic (C1–3) and Staphylococcal Protease (G4) Peptides Necessary To Determine the Whole Structure[a]

peptide composition	T2S	C1	C2	C3	G4
Cys (Cm)	2.9 (3)	1.2 (1)		2.0 (2)	2.2 (2)
Asx	4.2 (4)	3.9 (4)			
Ser	1.9 (2)	0.9 (1)		1.1 (1)	0.9 (1)
Glx	3.3 (3)	1.1 (1)		2.0 (2)	1.1 (1)
Pro	1.2 (1)	0.9 (1)			
Gly	2.3 (2)		0.8 (1)	1.2 (1)	0.9 (1)
Ala	6.0 (6)	2.8 (3)	1.0 (1)	1.9 (2)	1.7 (2)
Val	2.8 (3)	1.4 (2)[b]	1.1 (1)		
Ile	1.7 (2)	0.6 (1)		1.1 (1)	0.8 (1)
Leu	2.2 (2)	1.9 (2)			
Tyr	1.0 (1)		0.9 (1)	0.7 (1)	
Phe	2.0 (2)	0.9 (1)	0.9 (1)		
His	3.0 (3)		2.3 (2)	0.9 (1)	
Arg	0.9 (1)			1.0 (1)	0.8 (1)
total	(35)	(17)	(7)	(12)	(9)

[a] Values shown are molar ratios from acid hydrolysates without corrections and (within parentheses) the sums from the sequences. [b] Low recovery of Ile and Val is explained by the presence of an Ile–Val bond giving incomplete hydrolysis.

Upon chromatography of fractions 12–14 on CM-52-cellulose, 13 peaks were resolved (Figure 2). The major labeled peptide (fractions 50–53) is cationic and represents 60% of the total label applied to this column. The amino acid composition (Table I) of the peptide recovered shows three residues of *S*-(carboxymethyl)cysteine from hydrolysis of the amidated derivative and three residues of histidine. The minor peak of radioactivity at fraction 42 of the CM-52 eluate constitutes 17% of the total label applied but is impure. On repurification, the labeled component gives an amino acid composition and N-terminal sequence (manual DABITC–Edman degradation) identical with those of the major labeled peptide. Hence, the specificity of labeling is extensive and the two radioactive peaks contain a common structure.

Sequence Analysis. The results of sequence determination of the major [^{14}C]carboxamidomethyl peptide are summarized in Figure 3. Direct degradation in a modified liquid-phase sequencer revealed the 31 first residues and gave preliminary identification of the four last residues. The three derivatized

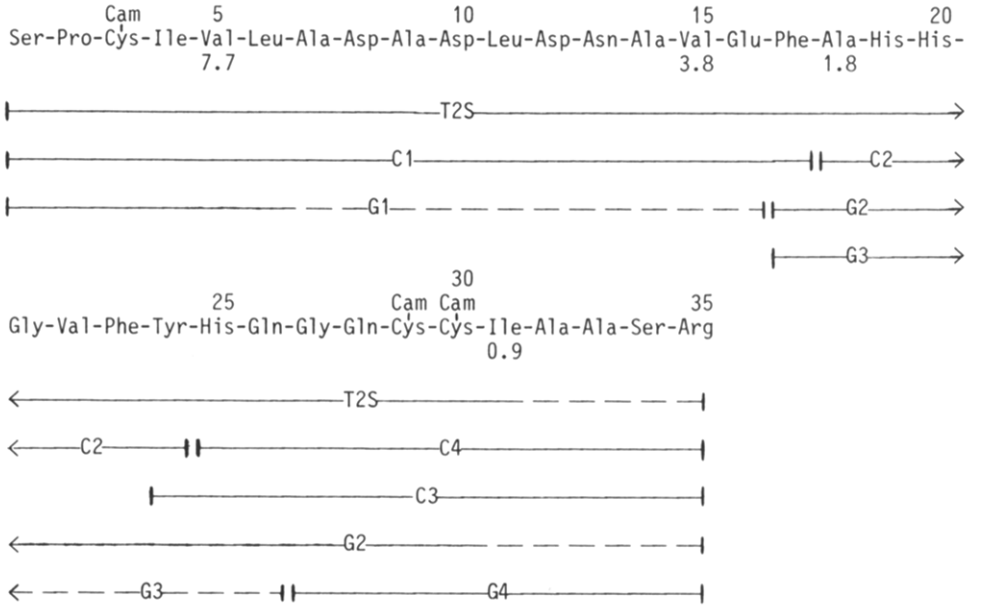

FIGURE 3: Amino acid sequence of the labeled carboxamidomethylated tryptic peptide of human liver aldehyde dehydrogenase E_1. Solid lines show parts of peptides determined by sequence analysis; dashed lines show the remaining parts of peptides purified. Peptides were obtained from redigestions with chymotrypsin (C) or Glu-specific staphylococcal protease (G) and analyzed by DABITC– and dansyl–Edman degradations. Cleavage on either side of Tyr-24 was nonstoichiometric, giving two sets of largely overlapping peptides. The parent tryptic peptide was analyzed by liquid-phase sequencer degradations (values show nanomoles recovered at the cycles indicated).

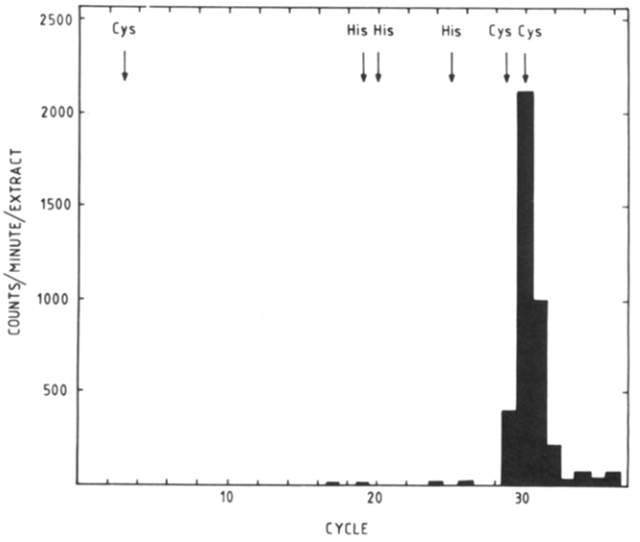

FIGURE 4: Radioactivity detected in extracts at each cycle of Edman degradation of the labeled tryptic peptide (starting material was about 50 000 cpm; cycle yields are without corrections for initial coupling or repetitive yield). Positions of Cys and His residues from Figure 3 are shown with arrows.

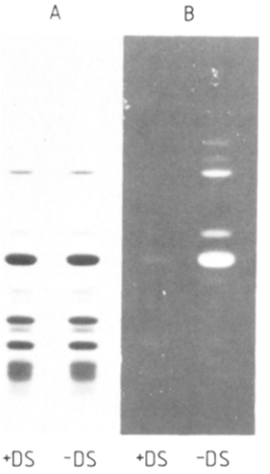

FIGURE 5: Blockage of carboxamidomethylation of liver aldehyde dehydrogenase by disulfiram, as revealed by $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr peptides. (A) CNBr fragments stained with Coomassie Blue. (B) Autoradiography of (A). +DS: E_1 (1.2 mg/mL) incubated with 0.022 mM disulfiram for 10 min. –DS: the same without disulfiram. Both samples were subsequently dialyzed overnight before treatment with 0.022 mM iodo[^{14}C]acetamide–0.7 mM NAD for 6.5 h. The two preparations were finally uniformly carboxymethylated in guanidine hydrochloride before CNBr cleavage, followed by electrophoresis in 16% polyacrylamide gels.

cysteine residues were found at positions 3, 29, and 30, and radioactivity measurements established that the majority of the label was present in cycle 30, with little detected in cycle 29 (Figure 4). This result identifies the labeled residue as a cysteine, and confirms the selectivity of the iodoacetamide reaction. The entire structure was confirmed by manual DABITC degradations of peptides from redigestions with chymotrypsin and staphylococcal protease. Particularly, a minor peptide (G4), presumably generated by cleavage at a deamidated Gln-26, was useful in ordering the last four residues. The specificity of labeling was also confirmed by autoradiography of the thin-layer plates from the manual degradations. Amino acid compositions of all peptides necessary to deduce the structure are given in Table I.

Disulfiram Blockage of Carboxamidomethylation. When iodo[^{14}C]acetamide was reacted with isoenzyme E_1 (1 mg/mL of phosphate buffer, pH 7) which had previously been exposed to disulfiram (equimolar amount in relation to enzyme subunits), only 15% as much label was incorporated as in the control containing no disulfiram, i.e., 0.16 vs. 1.06 mol/enzyme tetramer. These preparations, labeled in the absence and presence of disulfiram, were cleaved with CNBr and the resultant peptides were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis. Subsequent autoradiography gave the patterns shown in Figure 5. In the absence of disulfiram, one peptide band has nearly all the radioactivity (two minor, labeled, higher molecular weight fragments are partial cleavage products). In the presence of disulfiram no band is labeled, showing that the iodoacetamide alkylation is prevented by disulfiram.

Discussion

The results clearly establish one cysteine residue (at position 30 in Figure 3) as the reactive group in the region of liver aldehyde dehydrogenase selectively alkylated by iodoacetamide and protected by disulfiram against this reaction. The selectivity of the modification is shown by the stoichiometry of incorporation (Hempel & Pietruszko, 1981), recovery of label (Figure 4), and peptide mapping (Figures 1, 2, and 5), all supporting the conclusion. The selectivity is particularly important in view of an adjacent cysteine residue (Cys-29, Figure 3), one other cysteine residue (Cys-3), and three histidine residues (at positions 19, 20, and 25) in the same peptide, without labeling in these other residues. The selectivity is further emphasized by the nonreactivity of the remaining cysteine residues outside the region characterized.

Selective alkylation of single cysteine residues in dehydrogenases is common (Holbrook et al., 1967). Even Cys-Cys sequences occur in some of these enzymes but then outside the active site. For example, selective labeling at a Cys-Cys pair has been reported in yeast alcohol dehydrogenase (Balestrieri et al., 1975). In aldehyde dehydrogenase, the present results establish for the first time that two cysteine residues are adjacent. In addition, two further cysteine residues may not be too far away in the native conformation of the enzyme, since the labeled peptide has a third cysteine residue (Figure 3) and since the coenzyme binding site has still another cysteine residue (von Bahr-Lindström et al., 1981). The relative roles of all these residues is not established, but the one at position 30 is concluded to be important, since modification by iodoacetamide strongly affects catalytic activity and since the modification can be blocked by prior exposure of the enzyme to disulfiram (Figure 5).

The ability of disulfiram to block the selective alkylation of human aldehyde dehydrogenase isoenzyme E_1 suggests that the iodoacetamide-reactive cysteine-30 of the segment now characterized also is the residue reacting with disulfiram. This possibility is compatible with the facts that similar degrees of residual activity (5–10%) remain after full reaction with either reagent and that both reactions are stimulated by NAD^+ (Hempel et al., 1980; Hempel & Pietruszko, 1981; Kitson, 1981).

In view of the importance of thiol groups in aldehyde dehydrogenase, it is of interest to notice that the cysteine residue at position 29 may affect the reactivity of the cysteine at position 30, but this cannot be judged from the present results. Formation of a cystine disulfide bridge in the reaction with disulfiram has recently been suggested (Vallari & Pietruszko, 1982), but the residues involved were not determined. Another inactivation may be explained by the presently determined structure. Thus, the Cys-Cys sequence at positions 29–30 is consistent with inactivations using arsenosophenyl butyrate or arsenite (together with mercaptoethanol), which were considered to indicate vicinal thiols (Jakoby, 1963; Deitrich, 1967). It may further form the explanation for the effectiveness of British Anti-Lewisite (2,3-dimercaptopropanol) in reversing arsenite inactivation (Deitrich, 1967).

While the catalytic residue itself need not be the one alkylated by iodoacetamide or disulfiram [cf. Hempel & Pietruszko (1981) and Kitson (1981)], this possibility should not be entirely overruled. In fact, Wiseman & Abeles (1979) have concluded from competition experiments with cyclopropanone hydrate that iodoacetamide alkylates the catalytic residue of yeast aldehyde dehydrogenase. A further difficulty with the iodoacetamide reaction is that it is apparently complete at a stoichiometry (maximum of two per tetramer) less than the number of subunits. However, horse aldehyde dehydrogenases have been reported to function with half-of-the-sites reactivity (Weiner et al., 1976; Eckfeldt & Yonetani, 1976b; Takahashi & Weiner, 1980), and this could possibly be taken to support an iodoacetamide reaction with the catalytic residue, although evidence for half-of-the-sites reactivity was not observed with human isoenzyme E_1 (Vallari & Pietruszko, 1981). In any event, the segment now characterized closely influences the catalytic residue.

Finally, recent suggestions of the involvement of histidine and tyrosine in catalysis by aldehyde dehydrogenases (Takahashi et al., 1981) are also of interest. While the present results cannot judge any residue beyond Cys-30, the Tyr-His structure at positions 24 and 25 may be noted, as well as the His-His structure at positions 19 and 20.

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Kinetics and Equilibria of Active Site Core Extrusion from Spinach Ferredoxin in Aqueous *N,N*-Dimethylformamide/Triton X-100 Solutions†

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ABSTRACT: The nature and reactivity of each species participating in core extrusion of the [2Fe-2S] cluster of spinach ferredoxin by benzenethiol have been investigated in a novel aqueous medium containing 10-40% (v/v) *N,N*-dimethylformamide (DMF) plus 5-6% (v/v) of the nonionic detergent, Triton X-100. By use of visible absorption and circular dichroism spectroscopies, it is found that prior to addition of benzenethiol, modifications of the ferredoxin induced by DMF are reversible. Both the extent and rate of modification are dependent on DMF concentration and on ionic strength. At ferredoxin concentrations near 0.1 mM, complete modification by DMF is shown to be unnecessary for quantitative core extrusion provided that the benzenethiol concentration is $\gtrsim$50 mM and the DMF concentration is $\gtrsim$10% (v/v). When benzenethiol is added after DMF, core extrusion occurs in two phases. Our interpretation is that the rapid phase corresponds to core extrusion of that portion of the ferredoxin already modified by DMF and that the slower phase corresponds to modification of the remaining portion of the ferredoxin by DMF. When DMF solutions containing benzenethiol and Triton X-100 in various ratios are mixed with aqueous solutions of spinach ferredoxin, the rate of core extrusion appears to be determined both by the rate at which the ferredoxin is modified and by the Triton/benzenethiol ratio. Under all conditions examined we observe significantly faster rates of core extrusion from spinach ferredoxin in aqueous Triton/DMF than in 80/20 (v/v) hexamethylphosphoramide/water, a previously used core extrusion medium. Our results suggest a catalytic role for the micellar phase.

Core extrusion of the active centers of iron-sulfur proteins has proven to be a useful analytical tool for the determination of the type and number of [2Fe-2S] and [4Fe-4S] centers in both simple and complex iron-sulfur proteins (Gillum et al., 1977; Averill et al., 1978; Coles et al., 1979; Kurtz et al., 1979). The method is based on a reaction in which cysteine thiolate ligands to the iron-sulfur center in the holoprotein are replaced by PhS^-:

$$\text{holoprotein} + \text{PhSH} \rightarrow [Fe_nS_n(SPh)_4]^{2-} + \text{apoprotein}$$

where $n = 2, 4$. Distinctive features of the method include the use of high concentrations of organic solvent (80 vol %) in order to ensure adequate solubility of the added thiol and of $[Fe_nS_n(SPh)_4]^{2-}$ as well as unfolding of the proteins to a sufficient extent for the extrusion reactions to take place. High concentrations of added thiol are mandatory in order to prevent decomposition or dimerization of the extruded cluster.

Recently one of us (Kurtz, 1982) has devised a new method of core extrusion using much lower concentrations of organic solvent in the presence of the nonionic detergent Triton X-100. We have now investigated in further detail the nature and reactivity of each species participating in the active site core extrusion from spinach ferredoxin under these conditions.

Materials and Methods

Spinach ferredoxin was prepared essentially as previously described (Petering & Palmer, 1970) and stored at -24 °C in 0.15 M Tris-HCl,[1] pH 7.3, containing 1 M NaCl. Aliquots were desalted just before each experiment on a Sephadex G-25 fine column (0.8 × 12 cm) equilibrated with 50 mM Tris-HCl, pH 8.1. Apoprotein was prepared by treatment of the holoprotein with 10% trichloroacetic acid. The protein pellet was washed with 1% trichloroacetic acid and resuspended in the same buffer used for the native protein.

DL-Dihydrolipoic acid was prepared by $NaBH_4$ reduction of a methanolic solution of the oxidized form. The solution was then brought to pH 4.5 with HCl and dried in a rotary evaporator, and the reduced compound was dissolved in DMF. Molarity of this solution was assayed with 5,5′-dithiobis(2-

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[1] Abbreviations: DMF, *N,N*-dimethylformamide; Et, $-C_2H_5$; PhSH, benzenethiol; CD, circular dichroism; Fd, ferredoxin; Triton, Triton X-100; Tris, tris(hydroxymethyl)aminomethane.

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