Effects of Low Concentrations of Guanidine Hydrochloride on Pig Heart Lipoamide Dehydrogenase†

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ABSTRACT: The effects of low concentrations of guanidine hydrochloride (Gdn·HCl) on pig heart lipoamide dehydrogenase have been studied. Levels of Gdn·HCl below 1.0 M produce a rapid and reversible red shift in the visible spectrum of enzyme bound FAD. The magnitude of the difference spectrum, perturbed minus native enzyme, shows a hyperbolic dependence on Gdn·HCl concentration with a change in extinction coefficient at 501 nm of +1.2 mM^{-1} cm^{-1} at 1.0 M and an extrapolated value of +1.72 mM^{-1} cm^{-1} at infinite concentration of perturbant. Similar but much less intense difference spectra can be obtained by using urea. Gdn·HCl inhibits the catalysis of the NADH–lipoamide and dihydrolipoamide–acetylpyridine adenine dinucleotide reactions but slightly enhances the ability of lipoamide dehydrogenase to reduce 2,6-dichloroindophenol. This inhibition was shown to be reversible. The sluggish reactivity of the native enzyme toward 5,5′-dithiobis(2-nitrobenzoic acid) is markedly enhanced by Gdn·HCl. The pseudo-first-order rate constant, k_f, for the most reactive thiol is a linear function of denaturant concentration. The six remaining titratable cysteine residues are kinetically equivalent, with rate constants between 30- and 85-fold smaller than k_f depending on the concentration of perturbant. The enzyme exhibits similar behavior toward 6,6′-dithiodinicotinic acid. At these low levels, Gdn·HCl is not thought to effect sizable changes in gross conformation of the enzyme but rather localized perturbations *via* binding of denaturant molecules. Dissociation of FAD becomes significant at concentrations of Gdn·HCl greater than 1.0 M. Evidence is presented suggesting that the reactive thiol and the flavine binding site represent different regions of the enzyme.

Lipoamide dehydrogenase (EC 1.6.4.3), as isolated, is composed of two polypeptide chains each binding one molecule of FAD (Massey *et al.*, 1962). The FAD bound to the native enzyme exhibits a highly resolved visible spectrum indicative of a strong interaction between cofactor and apoenzyme in an apolar environment (Palmer and Massey, 1968). The cystine residue which participates directly in electron transfer is in redox contact with the FAD (Massey and Veeger, 1961). The hydrophobic residues adjacent to this cystine residue have been postulated to interact with dihydrolipoate positioning it for interchange (Burleigh and Williams, 1972; Williams and Arscott, 1972). Evidence indicates that the oxidized enzyme contains two distinct pyridine nucleotide binding sites and that NAD^+ in both these sites interacts with the FAD (Massey and Veeger, 1961; Su and Wilson, 1971; van Muiswinkel-Voetberg and Veeger, 1973a; R. G. Matthews and C. H. Williams, 1974, submitted). Thus the active site region of this enzyme must be quite extensive.

Pig heart lipoamide dehydrogenase contains 10 half-cystines per FAD two of which comprise the active site cystine; 7 of the remaining 8 are titratable with DTNB[1] in 5 M Gdn·HCl, while the remaining half-cystine is very unreactive (R. G. Matthews, L. D. Arscott, and C. H. Williams, 1974, submitted). Only one of the 7–8 thiols in the native enzyme is appreciably reactive with DTNB. The oxidation of two thiols to form a cystine residue is catalyzed by cupric ions and leads to the loss of most of the NADH/Lip(S-S) activity and a marked increase in diaphorase activity (Veeger and Massey, 1962; Casola *et al.*, 1966). The results of R. G. Matthews and C. H. Williams (1974, submitted) indicate that a further cysteine residue is at or very near one of the NAD^+ binding sites. The thiols of lipoamide dehydrogenase are therefore of interest since their modification results in large changes in enzymatic activity.

It has been observed[2] that low concentrations of Gdn·HCl cause a general shift of the enzyme bound flavine spectrum to longer wavelengths in both pig heart and *Escherichia coli* enzymes. This perturbation is reversible and yields a characteristic difference spectrum easily differentiated from that resulting from dissociation of FAD from the holoenzyme. The difference spectrum was observed to be quite similar to the temperature difference spectra recently reported by Müller *et al.* (1973) but is much more intense.[2]

We have examined the effects of relatively low concentrations of Gdn·HCl on the spectroscopic properties, the catalytic activities, and the thiol residues of pig heart lipoamide dehydrogenase.

Materials and Methods

Pig heart lipoamide dehydrogenase was obtained from Boehringer and was further purified using a calcium phosphate gel-cellulose column, essentially as described for the *E. coli* enzyme (Williams *et al.*, 1967). Approximately 35% of the absorbance at 280 nm of the commercial material is due to colorless contaminating proteins. Concentrations of enzyme are expressed with respect to flavine, using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm (Massey, 1960a).

DTNB was obtained from Calbiochem, 2,2′-dithiodipyridine

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[1] Abbreviations used are: APAD, acetylpyridine adenine dinucleotide; DCI, 2,6-dichloroindophenol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn·HCl, guanidine hydrochloride; $Lip(SH)_2$ and Lip(S-S), reduced and oxidized lipoamide respectively; TNB, 5-thio-2-nitrobenzoate.

[2] Unpublished observations of Mr. L. D. Arscott, V. A. Hospital, Ann Arbor, Mich.

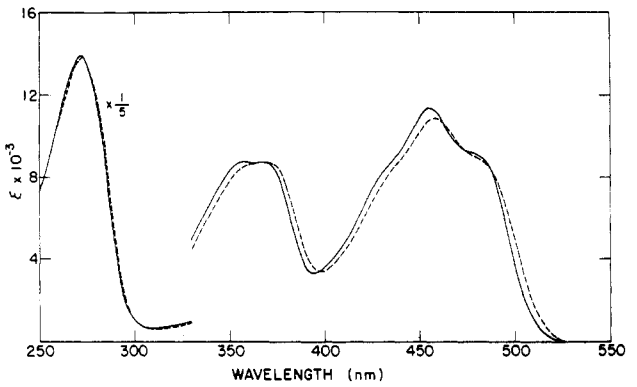

FIGURE 1: Spectrum of pig heart lipoamide dehydrogenase at 25°, in 100 mM phosphate buffer (pH 7.6) with 0.3 mM EDTA in the presence of either 1 M NaCl (——) or 0.7 M Gdn·HCl plus 0.3 M NaCl (- - - - -).

from Aldrich, and 6,6′-dithiodinicotinic acid from Newcell. Ultrapure grade guanidine hydrochloride and urea were from Mann.

Unless otherwise stated, phosphate buffer is 100 mM, pH 7.6 and contains 0.3 mM EDTA. Mixtures of 2 M solutions of NaCl or Gdn·HCl were used to prepare the stock perturbants. Routinely, experiments were performed at constant ionic strength (at a chloride ion concentration of 1 M). Since the pH of phosphate buffer is dependent on ionic strength, the pH of the perturbants was adjusted to give the correct pH to the final mixture in the cuvet. Solutions were clarified by Millipore filtration where necessary.

Difference Spectra. Enzyme was diluted by the addition of an equal volume of perturbing solution to the cuvet with stirring to avoid locally high concentrations of denaturant. Spectra were recorded in a Cary 14 or 118 C spectrophotometer using matched cells with reference and sample cuvets containing 1 M NaCl and Gdn·HCl–NaCl mixtures, respectively. Base lines were recorded minus enzyme. When necessary, dry air was forced through the cell compartment to avoid condensation.

Stopped Flow. The experiments were performed with Dr. D. Ballou, University of Michigan, using an improved version of the instrument described by Gibson and Milnes (1964). Enzyme was mixed in 2–3 msec at 16° with an equal volume of perturbant and the subsequent changes in flavine spectrum at 501, 449, and 384 nm were followed. The final spectrum of the mixed solution, containing 3.14 μM enzyme and 0.4 M Gdn·HCl, was constructed by taking absorbance readings at 10-nm intervals.

Circular Dichroism (CD) Spectra. A Jasco ORD/CD spectrometer was used to record CD spectra, with enzyme of either 4.8 μM (in phosphate buffer) or 2.4 μM (in solutions containing 1 M NaCl or 0.4 M Gdn·HCl) in cells having a path length of 1 cm.

Fluorescence Spectra. Fluorescence emission spectra were obtained using an instrument designed and built by Dr. D. Ballou. Spectra were obtained using 6.6 μM enzyme, exciting at 365 nm.

Catalytic Activities. NADH/Lip(S-S) assays were performed at pH 6.3 essentially as described by Massey (1960b) using a 3-ml volume containing 0.05 M phosphate buffer, 2 mg of bovine serum albumin, 0.3 μmol of NADH, 0.2 μmol of NAD^+ and 2 μmol of D,L-lipoamide. $Lip(SH)_2$/APAD activity was measured at pH 7.6 as described by Williams (1965). NADH/DCI assays were conducted at pH 7.5 following the procedure of Casola *et al.* (1966). Where necessary, these three assay procedures were modified to include appropriate amounts of Gdn·HCl and NaCl.

Sulfhydryl Reactivity. Generally, solutions of perturbants and the sulfhydryl reagent were mixed and the pH was adjusted so that the addition of enzyme would give the appropriate conditions in a total volume of 1.5 ml. Two aliquots of this solution minus enzyme (usually 1.3 ml) were equilibrated in the spectrophotometer and equal volumes of buffer and enzyme were added to reference and sample cuvets, respectively, to start the reaction.

Stock solutions of DTNB, 6,6′-dithiodinicotinic acid, and 2,2′-dithiodipyridine were prepared in phosphate buffer and were stored in the dark at 4°. The solutions were standardized spectrophotometrically by the addition of a small aliquot to a volume of buffer containing an excess of cysteine, dithiothreitol, or sulfite. Extinction coefficients of 13.6 mM^{-1} cm^{-1} at 412 nm for the TNB anion (Beutler *et al.*, 1963), and 7.06 mM^{-1} cm^{-1} at 343 nm for 2-thiopyridone (Grassetti and Murray, 1967) were used. The extinction coefficient of the thiolate anion of 6,6′-dithiodinicotinic acid was determined by the addition of aliquots of a standardized solution of dithiothreitol (*ca.* 20 mM in phosphate buffer adjusted to pH 5.5) to an approximately millimolar solution of the disulfide in phosphate buffer. The value of 10.5 ± 0.2 mM^{-1} cm^{-1} obtained at 344 nm is slightly higher than that used by Ando and Steiner (1973). The thiolate species were examined for their stability under the conditions employed by adding a very dilute solution of cysteine (instead of enzyme) to the sample cuvet. A small correction was made for the decay of the thionitrobenzoate anion, which was only significant in affecting the slow phase of the reaction of DTNB with the enzyme. It was also found that the reoxidation of the TNB anion yielding DTNB is markedly enhanced by light at normal laboratory levels.

Results

Optical Properties. FAD bound to native pig heart lipoamide dehydrogenase exhibits a highly resolved visible spectrum indicative of a strong interaction between cofactor and apoenzyme (Figure 1). Concentrations of guanidine hydrochloride below 1.0 M cause a general shift of this spectrum to longer wavelengths, together with a decrease in absorption of the 450-nm peak, a slightly less pronounced shoulder at 480 nm, and a shift in the relative intensities of the two maxima centered around 360 nm (Figure 1). This perturbation is complete within the time taken to mix and begin to record a spectrum. Removal of the Gdn·HCl by dialysis restores the spectrum to that of the native enzyme. Further, the effect is not simply due to increased ionic strength since the visible spectrum of lipoamide dehydrogenase in 1 M sodium chloride only deviates from that of the native enzyme by a slightly increased absorption around 380 nm ($\Delta\epsilon_{383}$ = 150 M^{-1} cm^{-1}). Unless otherwise stated, the experiments described below were conducted at constant ionic strength by the addition of NaCl to a concentration of 1 M in chloride ion.

Figure 2 shows the perturbation of the flavine chromophore by Gdn·HCl as difference spectra using native enzyme in 1 M NaCl as reference. Spectra in 0.2, 0.4, 0.7, and 1.0 M Gdn·HCl show a very similar general form with identical principal maxima at 501, 449, 425, and 384 nm. However, differences do occur, for example, in the noncoincidence of the points of intersection of the base line around 370 nm. This may reflect, in part, errors in the dilution of the enzyme by the perturbing solutions, since these will introduce sizable distortions of the base line at strongly absorbing regions of the flavine spectrum. A double reciprocal plot of $\Delta\epsilon_{501}$ *vs.* Gdn·HCl concentration is linear, yielding a limiting value at infinite concentration of guanidine of $\Delta\epsilon_{501}$ = 1.72 ± 0.05 mM^{-1}. The difference

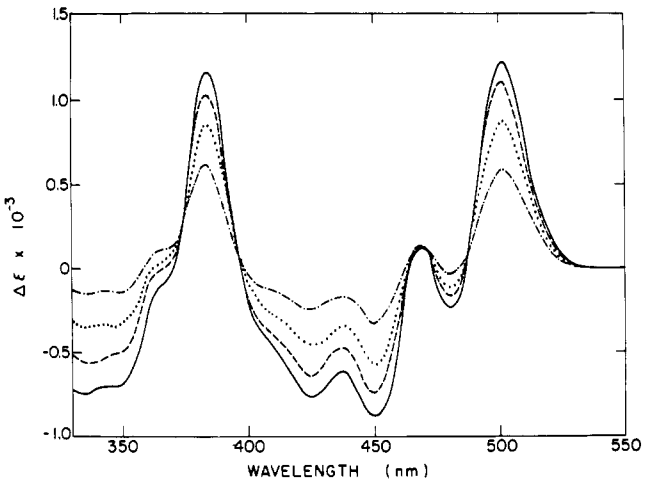

FIGURE 2: Difference spectra of lipoamide dehydrogenase (33.2 μM) at 25° in 100 mM phosphate buffer (pH 7.6) with 0.3 mM EDTA as a function of Gdn·HCl concentration: (·····) 0.2 M; (····) 0.4 M; (- - - -) 0.7 M; and (——) 1.0 M Gdn·HCl. NaCl was added to the sample cuvet to give a final concentration of chloride ion of 1 M; the reference cuvet contained 1 M NaCl.

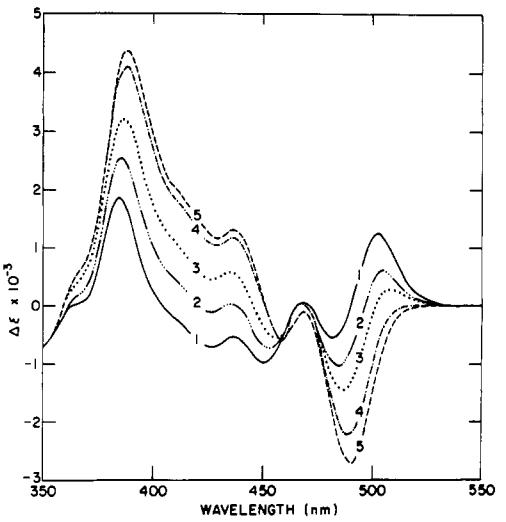

FIGURE 3: The change in difference spectrum of lipoamide dehydrogenase in 1.43 M Gdn·HCl with time. The conditions were as in Figure 2, except that the enzyme was 6.56 μM and the reference cuvet contained 1.43 M NaCl. Spectra were recorded at 0.5 nm/sec and were commenced 2.5, 28, 72, 244, and 530 min after mixing (curves 1, 2, 3, 4, and 5, respectively).

spectrum in the ultraviolet region displays a maximum at 288 nm and a minimum at 270 with crossover points at 304, 277, and 252 nm. At 0.4 M Gdn·HCl $\Delta\epsilon_{288}$ and $\Delta\epsilon_{270}$ values are +1.2 and −0.77 mM^{-1} cm^{-1}, respectively. Slight shoulders are apparent at 283 and 277 nm on the positive peak and at 260 nm on the negative limb. Interpretation of these difference spectra is complicated since both flavine and the aromatic residues of the protein contribute to the 272-nm absorption maximum of the holoenzyme (Figure 1).

If the above experiment is performed without the addition of sodium chloride to maintain constant ionic strength a plot of $\Delta\epsilon_{501}$ *vs.* Gdn·HCl concentration is not now a hyperbola. Increasing ionic strength enhances the magnitude of the difference spectrum obtained at a given concentration of guanidine. The deviation between the two sets of data is therefore most marked at low concentrations of perturbant. Thus at 0.2 M Gdn·HCl the ratio of $\Delta\epsilon_{501}$ measured in the presence and absence of salt is 2.9, whereas at 0.4 M Gdn·HCl it is 1.8.

In contrast to the difference spectrum of the enzyme, that of free FAD in phosphate buffer induced by 1.0 M Gdn·HCl is rather featureless and of low intensity, consisting of two broad positive maxima at 440 and 385 nm with extinctions of approximately 150 and 200 M^{-1} cm^{-1}, respectively.

The CD spectrum of the enzyme in 1.0 M NaCl and in 0.4 M Gdn·HCl show very minor differences between 450 and 300 nm, and are very similar to that obtained in phosphate buffer alone (van Muiswinkel-Voetberg and Veeger, 1973b; Brady and Beychok, 1969). The positive CD maximum at 370 nm is therefore insensitive to the presence of these salts. Similarly, the fluorescence emission spectrum of enzyme-bound FAD (Casola *et al.*, 1966) measured in phosphate buffer is not influenced markedly by the presence of molar NaCl or 0.4 M Gdn·HCl. Compared to the spectrum in salt alone, 0.4 M Gdn·HCl produces an approximately 5% increase in fluorescence emission at 520 nm and 22°.

The difference spectrum of the enzyme shows reversible increases in magnitude with decreasing temperature amounting to 50% when both sample (0.4 M Gdn·HCl) and reference cuvets are cooled from 25 to 3°. No new features in the spectrum appear.

Perturbations in the visible spectrum of lipoamide dehydrogenase produced by guanidine hydrochloride occur very rapidly. The changes in absorbance at 501, 449, and 384 nm, produced on mixing enzyme and perturbant in a stopped-flow apparatus at 16° to give a final concentration of 0.4 M Gdn·HCl, were complete in 2–3 msec.

The modified spectra produced by concentrations of Gdn·HCl below 0.7 M Gdn·HCl remain unchanged over 1 hr at 25°. The enzyme is, however, unstable in Gdn·HCl concentrations of 1.0 M and above, with the associated changes in difference spectrum being illustrated in Figure 3 for 1.43 M perturbant. The process represents a release of flavine from the protein, since the final spectrum closely resembles that of FAD in 1.4 M Gdn·HCl minus native enzyme in 1.4 M NaCl. A semilogarithmic plot of the changes in absorbance at 501 nm *vs.* time is concave downwards consisting of a fast phase followed by a more smoothly curved slower phase. A comparison of the times required for the dissociation to be half-complete in 1.71, 1.43, and 1.0 M Gdn·HCl of 10 min, 1 hr, and 24 hr, illustrates the sensitivity of the reaction to perturbant concentration.

Catalytic Activities. Figure 4 shows variation of three catalytic activities of lipoamide dehydrogenase with Gdn·HCl concentration at constant ionic strength. For comparison, the changes in $\Delta\epsilon_{501}$ of the difference spectrum, expressed as a percentage of the value extrapolated to infinite Gdn·HCl concentration, are plotted on the same graph. The turnover numbers in the NADH/Lip(S-S) and $Lip(SH)_2$/APAD assays show a smooth decline with increasing concentrations of perturbant, with 18 and 9% of the original activity remaining at 1.0 M Gdn·HCl, respectively. In contrast, the diaphorase activity using DCI as acceptor increases. It should be noted that the turnover numbers obtained in 1.0 M NaCl (100% in Figure 4) differ from the comparable values in the standard assays using low ionic strengths. Expressed as percentages of the routine assays, the values in salt are: NADH/Lip(S-S), 80%; $Lip(SH)_2$/APAD, 38%; and NADH/DCI, 152%.

The inhibition of the activity of the enzyme by low concentrations of Gdn·HCl was reversible. A NADH/Lip(S-S) assay was performed in 0.2 M Gdn·HCl for 30 sec, to establish an initial rate, and then a portion was removed and diluted sixfold into fresh assay medium containing 1 M NaCl. Within experimental error, the enzyme regained the turnover number predicted for 0.033 M Gdn·HCl.

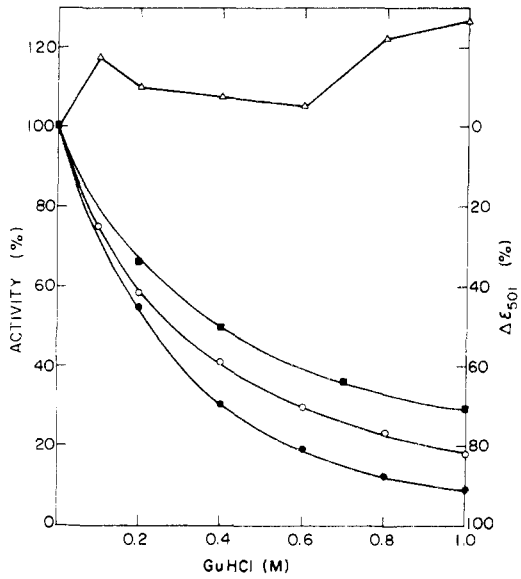

FIGURE 4: Catalytic activities and changes in difference spectrum of lipoamide dehydrogenase as a function of Gdn·HCl concentration, expressed as percentages of their values in 1 M NaCl. Assay conditions are described in Methods: (△) NADH/DCI; (○) NADH/Lip(S-S); and (●) $Lip(SH)_2$/APAD. The per cent change in difference spectrum (■) has been calculated from the $\Delta\epsilon_{501}$ values of Figure 2.

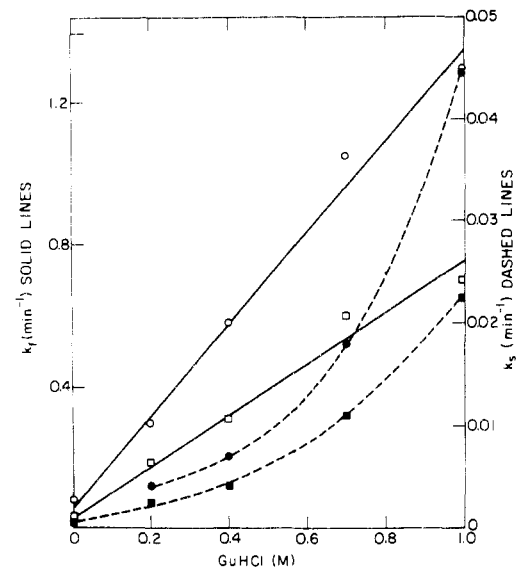

FIGURE 6: The dependence of the pseudo-first-order rate constants on Gdn·HCl concentration for the fast and slow phases shown in the reaction of lipoamide dehydrogenase with DTNB (circles) or 6,6′-dithiodinicotinic acid (squares). The conditions using DTNB are those described in the legend to Figure 5. 6,6′-Dithiodinicotinic acid was used at 1.28 mM with an enzyme concentration of 7.58 μM. The treatment of the results is described in the text.

Reactivity of Thiol Groups. Lipoamide dehydrogenase from pig heart has been previously shown to contain seven cysteine residues titratable with DTNB in 5 M Gdn·HCl. The native enzyme in 1.0 M NaCl reacts very sluggishly with this reagent (Figure 5). Between 0.2 and 1.0 M Gdn·HCl the production of thiolate anion is more rapid and is clearly biphasic with one cysteine reacting in the fast phase. At 1.0 M Gdn·HCl the reaction can be resolved satisfactorily into two pseudo-first-order processes with rate constants of k_f (0.94 thiols), 1.25 min^{-1}, and k_s (5.8 thiols), 0.045 min^{-1}. At lower concentrations of perturbant, greater uncertainty in the extent of decay of the thiolate anion (see Methods) and a tendency for the protein to precipitate made analysis of the slower phase difficult. However, by using a linear extrapolation, the fast phases for the other concentrations of Gdn·HCl were shown to be first order in enzyme. The resulting values of k_f increase linearly with increasing concentration of perturbant (Figure 6). Very similar values of k_f are obtained if the slow phase is assumed to proceed exponentially to the same end point as that in 1.0 M Gdn·HCl, and k_f and k_s are varied to achieve the best fit to the experimental trace. In contrast to the behavior of k_f, a plot of k_s *vs.* Gdn·HCl is markedly concave upwards (Figure 6). Lowering the temperature to 3° did not significantly alter the ratio of k_f to k_s in 0.4 M Gdn·HCl and it was therefore impossible to further separate the fast and slow phases on this basis.

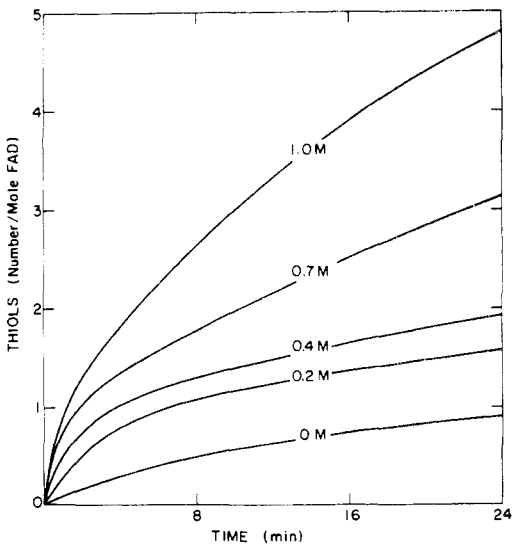

FIGURE 5: The reaction of lipoamide dehydrogenase with DTNB at 25°. Absorbance values have been converted into the number of cysteine residues modified by using an extinction coefficient for the thiolate anion of 13.6 mM^{-1} cm^{-1} at 412 nm (a small correction was applied, as detailed in the Methods, for the decay of TNB under the experimental conditions). The sample cuvet contained 7.44 μM enzyme, 1.36 mM DTNB, 0.3 mM EDTA, and the concentration of Gdn·HCl indicated in the figure (together with the NaCl required to maintain constant ionic strength) in a total volume of 1.5 ml of 100 mM phosphate buffer (pH 7.6). Enzyme was omitted from the reference cuvet.

Two analogous reagents 2,2′-dithiodipyridine and 6,6′-dithiodinicotinic acid exhibit a similar biphasic reaction with pig heart lipoamide dehydrogenase. Values of k_f obtained from the reaction of 6,6′-dithiodinicotinic acid with the enzyme show an approximately linear dependence on Gdn·HCl concentration, in contrast to the behavior of k_s for the remaining six cysteines (Figure 6).

Urea as a Denaturant. Massey *et al.* (1962) have reported that urea is an ineffective denaturant for oxidized pig heart lipoamide dehydrogenase. Thus over 70% of the activity of the enzyme was retained after storage for 1 month at 0° in 6.5 M urea. The solubility, spectrum, and sedimentation coefficient of the protein were similar to those of the untreated enzyme. We have measured the difference spectrum of lipoamide dehydrogenase produced by 5.4 M urea in phosphate buffer (pH 7.6). The observed spectrum is similar to that induced by Gdn·HCl, but with maxima occurring at wavelengths approximately 3 nm lower than those shown in Figure 2. However, in 5.4 M urea the perturbation of the flavine chromophore ($\Delta\epsilon_{499}$ = 600 M^{-1} cm^{-1}) is approximately equivalent to that produced by 0.2 M Gdn·HCl. Urea is similarly ineffective as a perturbant in the reaction of the enzyme with DTNB. No significant enhancement of the reactivity of the native enzyme toward this reagent occurs in the presence of 5.4 M urea.

Discussion

Guanidine hydrochloride is widely used as a denaturant although its mode of action is imperfectly understood (Tanford, 1970). A direct interaction of Gdn·HCl with proteins (Gordon, 1972) is thought to occur *via* two main mechanisms (Lee and Timasheff, 1974): firstly by hydrogen bond formation between adjacent peptide units of the polypeptide backbone, and secondly by lowering the free energy of transfer of hydrophobic moieties, principally aromatic side chains, from apolar regions to the aqueous milieu. Appreciable numbers of Gdn·HCl molecules have been shown to be associated with proteins at concentrations of perturbant considerably below that required to effect gross disruption of the protein *via* the cooperative denaturation process (Gordon, 1972; Lee and Timasheff, 1974). Each such binding may produce small localized perturbations of the protein (Weber, 1972) with a disruption of those forces which, in summation, contribute to the gross conformation of the polypeptide in its "native" state (Ikai *et al.*, 1973).

The perturbation of the visible spectrum of lipoamide dehydrogenase by low concentrations of Gdn·HCl is not thought to represent a substantial change in the gross conformation of the protein. Thus the CD and fluorescence spectra are similar to those of the native enzyme, the perturbation of the flavine chromophore occurs very rapidly, and the ultraviolet difference spectrum fails to reveal large changes in the environment of tyrosine and tryptophan residues. However, binding of denaturant may produce minor perturbations of the active site region of the enzyme with concomitant modification of the flavine spectrum and catalytic activity. This perturbation could include contributions from small conformational changes and from "solvent effects" on the FAD chromophore.

It should be noted that rather similar difference spectra can be obtained by using urea or by raising the temperature of the native enzyme (Müller *et al.*, 1973). Urea is recognized as an inferior denaturant to Gdn·HCl and, on a molar basis, is much less effective at perturbing the FAD chromophore in lipoamide dehydrogenase. Similarly, a 20° rise in temperature produces a maximum change in extinction coefficient of only 200 M^{-1} cm^{-1} in the visible spectrum. Several flavoproteins have been shown to undergo temperature-dependent conformational changes, *e.g.*, D-amino acid oxidase (Massey *et al.*, 1966), glutathione reductase, and lipoamide dehydrogenase (Veeger *et al.*, 1971). Further, the substantial decrease in fluorescence of FAD bound to lipoamide dehydrogenase on heating has been explained on the basis of the increased ability of the prosthetic group to adopt a quenched configuration, once a hydrogen bond to the adenine moiety has been broken by thermal energy (Palmer and Massey, 1968). It might be expected therefore that treatment with Gdn·HCl, like increasing temperature, would quench the flavine fluorescence. This is not the case (see Results).

Rajagopalan *et al.* (1961) have studied the inhibition of enzymes by urea and Gdn·HCl. The competitive inhibition observed in many cases was thought to involve binding of molecules of denaturant to the active site region of the enzymes. The results obtained in the present study must be interpreted with caution since the native enzyme itself undergoes marked changes in catalytic activity in molar sodium chloride. However, the decline in NADH/Lip(S-S) and $Lip(SH)_2$/APAD activities correlates loosely with the spectral changes (Figure 4), suggesting a common perturbation of the active site.

Kalse and Veeger (1968) have described a preparation of monomeric lipoamide dehydrogenase which exhibits a 20-fold enhanced diaphorase activity using DCI, with a lowered physiological activity. Under certain conditions the native dimer is reported to exist in equilibrium with the monomer. However, the effects reported in this study cannot be explained by a reversible monomerization induced by Gdn·HCl. Such an equilibrium would be expected to be shifted toward the monomer on lowering the enzyme concentration, whereas the $\Delta\epsilon_{501}$ values obtained in 0.4 M Gdn·HCl are independent of protein concentration between 33 and 10 μM. In addition, dimerization of the DCI-active monomer occurs relatively slowly (Kalse and Veeger, 1968), whereas the inhibition of the enzyme by 0.2 M Gdn·HCl is rapidly reversed on sixfold dilution into fresh assay mixture minus perturbant. A rapid dimerization would require a very large value for the rate constant since the diluted enzyme is approximately 3×10^{-10} M.

The biphasic spectral changes accompanying the release of FAD from the perturbed holoenzyme at the higher concentration of Gdn·HCl proceed without the appearance of spectroscopically distinct intermediates. Possibly the faster phase represents a reversible dissociation of flavine from the perturbed enzyme with a subsequent slower modification of the apoenzyme rendering it unable to recombine with FAD. Brady and Beychok (1969) used dialysis of lipoamide dehydrogenase against 1.5 M Gdn·HCl (pH 7.6) for 2 days at 2° to prepare their dimeric apoenzyme and found that rapid regeneration of holoenzyme required incubation with dithiothreitol. In contrast, the monomeric apoenzyme prepared by the acid $(NH_4)_2SO_4$ precipitation procedure of Kalse and Veeger (1968) recombines rapidly with FAD to yield a DCI-active monomer which subsequently dimerizes more slowly.

Generally, difference spectroscopy may provide a convenient sensitive means of following the release of flavine in the preparation of apoenzyme by Gdn·HCl treatment.

The dependence of k_f on Gdn·HCl concentration in the reaction of lipoamide dehydrogenase with DTNB can be explained by an increased conformational motility of the polypeptide in the vicinity of the reacting thiol. It must be noted that the observed rate of reaction could reflect very small equilibrium concentrations of a conformation about the thiol capable of reacting rapidly with DTNB. A rate-limiting unfolding of the protein is not responsible for the observed fast phase since k_f is DTNB dependent (unpublished results). There is some evidence to suggest that the reactive cysteine residue is not situated in the active center region of lipoamide dehydrogenase. In contrast to the hyperbolic dependence of $\Delta\epsilon_{501}$ and the changes in enzymatic activity in the NADH/Lip(S-S) and $Lip(SH)_2$/APAD assays, the k_f values are linear functions of Gdn·HCl concentration over the range studied. The monolabeled TNB–protein is active and the bound label does not appear to perturb the flavine chromophore markedly in the visible region (unpublished results). Further, 5.4 M urea effects a perturbation of the FAD chromophore roughly equivalent to 0.2 M Gdn·HCl without a corresponding enhancement of the native enzyme's reactivity with DTNB.

The aim of current work is to isolate a peptide containing the rapidly reacting cysteine residue in order to investigate its possible identity with half-cystines previously implicated in the inactivation of the enzyme with cupric ions.

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Flavine–Protein Interactions in Flavoenzymes. Effects of Aggregation of the Apoprotein of *Azotobacter* Flavodoxin on Coenzyme Binding†

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ABSTRACT: The effects of apoprotein concentration and buffer pH, concentration, and type on the kinetics and thermodynamics of the binding of flavine analogs to *Azotobacter* apoflavodoxin have been studied. The affinities of 3-methylflavine mononucleotide (3-MeFMN) and riboflavine were found to increase approximately tenfold when the concentration of apoprotein was decreased from 10^{-6} to 5×10^{-8} M. The shapes of the pH-affinity curves were also observed to be dependent on apoprotein concentration. At high apoprotein concentrations, the affinity of 3-MeFMN was the same in both phosphate-acetate and pyrophosphate buffers, whereas at low concentrations, the affinity in pyrophosphate buffer was higher than it was in phosphate-acetate. In order to explain the above results, it is proposed that the apoprotein exists in an equilibrium between monomer and polymer and that the affinity of flavine derivatives to monomer is higher than to polymer. The extent of polymerization is assumed to be a function of pH and buffer type. We have also observed that the binding velocity of 3-MeFMN to apoprotein was unaffected by the protein concentration. Thus, it can be concluded that the binding rates of flavine to monomer and to polymer are the same. The primary effect of apoprotein polymerization must therefore be on the rate constant for the release of flavine from the holoprotein. However, the binding velocities were found to be a function of buffer type and concentration. This must be due to a direct modification of the apoprotein-flavine interaction and not to a change in the equilibrium constant for the monomer-polymer reaction.

Previous work in this laboratory (Edmondson and Tollin, 1971a,b; Edmondson *et al.*, 1972; Barman and Tollin, 1972; D'Anna and Tollin, 1971, 1972; MacKnight *et al.*, 1973) has provided information on the kinetics, thermodynamics and pH dependence of the binding of flavine analogs to the apoprotein of various flavodoxins, particularly that derived from *Azotobacter*. However, very little information is available concerning the possibility of protein-protein interactions in these enzymes. In the earlier work, it was assumed that the flavodoxins have no subunit structure, mainly based on hydrodynamic measurements of the holoprotein of *Azotobacter* flavodoxin (Edmond-

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