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Mechanism of Formation and Quantitation of Imines, Pyrroles, and Stable Nonpyrrole Adducts in 2,5-Hexanedione-Treated Protein

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SUMMARY

The condensation of γ-diketones with protein ε-amino moieties to yield alkylpyrrole adducts has been demonstrated in many *in vitro* and *in vivo* systems, although certain features of this reaction remain unclear. The present *in vitro* study was designed to examine additional aspects of γ-diketone-protein interactions, including the possible formation of imine intermediates and stable nonpyrrole products, and the potential for conformational changes in pyrrolylated protein. Values for total, stable covalent binding were consistently higher than *p*-dimethylaminobenzaldehyde (DMAB)-detectable pyrrole adduct concentrations when bovine serum albumin (BSA) was incubated (24 hr, 37°C) with [^{14}C]-2,5-hexanedione (2,5-HD) at diketone:lysine ratios ≥5:1 (at pH 9.5) or 1:1 (at pH 7.4). Treatment of pyrrolylated BSA with proteases before the DMAB assay decreased but did not eliminate the difference between these parameters. Quantitative amino acid analysis of pyrrolylated BSA revealed molar decreases in lysine content equivalent to DMAB-detectable pyrrole adduct concentrations; no other amino acids were significantly altered. Cleavage of disulfide bonds in pyrrolylated BSA by dithiothreitol resulted in an apparent decrease in DMAB-detectable pyrrole, which was reversible upon subsequent protease treatment. A similar decrease was not seen with pyrrolylated concanavalin A, a protein that lacks disulfide linkages. Samples of BSA were incubated with [^{14}C]-2,5-hexanedione for 2–144 hr and a portion of each incubation mixture treated with $NaCNBH_3$ to selectively reduce imines to stable amines. Substantial levels of an imine intermediate were detected at 2, 6, and 24 hr but not at 144 hr. The above findings support proposed mechanisms involving imine intermediates in the pyrrolylation reaction. In addition, evidence for the formation of stable nonpyrrole adducts at high diketone:amine molar ratios has been provided. Results consistent with potential conformational alterations in pyrrolylated protein have also been demonstrated.

The reaction of γ-diketones with primary amines to yield 1,2,5-trisubstituted pyrroles (Paal-Knorr synthesis) was first described in the late 1900s (1, 2). Almost a century later, the human neurotoxic potential of the γ-diketones was revealed, as a consequence of exposure to the industrial solvents *n*-hexane and methyl *n*-butyl ketone (3). Both solvents are metabolized to 2,5-HD, the γ-diketone that is believed to be the actual neurotoxic species in this syndrome (3). Although pyrrolylation of protein after *in vitro* and *in vivo* 2,5-HD exposure has been demonstrated (4–6), many aspects of this reaction remain unclear.

Until recently, the molecular mechanism of the Paal-Knorr reaction was obscure, although early workers proposed schemes based on imine intermediates (7, 8) (Fig. 1). This was confirmed by Katritzky and coworkers by means of ^{13}C-, ^{1}H-, and ^{15}N-NMR spectroscopy of equimolar 2,5-HD:amine mixtures in $CDCl_3$ (9). Two rate-limiting steps were demonstrated; the initial intermolecular nucleophilic attack of amine nitrogen on carbonyl carbon and the subsequent intramolecular attack of enamine nitrogen on the second carbonyl function. These studies eliminated other suggested mechanisms involving the participation of dihydroxypyrrolidine species in the reaction (9).

A similar imine intermediate was proposed for the formation of 2,5-dialkylpyrrole adducts in protein exposed to γ-diketones under aqueous conditions, although no data were reported in support of this mechanism (5). Nevertheless, imine intermediates would also be likely in this case, considering their well-characterized formation in the reaction of protein amines with monoketones (10). A more important question concerns the relative rates of formation and disappearance of such an intermediate in 2,5-HD-treated protein, since these rates would determine whether significant levels of imine are present at any given time. A long-lived imine intermediate could conceiv-

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ABBREVIATIONS: BSA, bovine serum albumin; Con A, concanavalin A; DMAB, *p*-dimethylaminobenzaldehyde; DTT, dithiothreitol; 2,5-HD, 2,5-hexanedione; NF, neurofilament; PITC, phenylisothiocyanate; pyrrole-BSA, pyrrolylated BSA; pyrrole-Con A, pyrrolylated Con A; SDS, sodium dodecyl sulfate; TPCK-trypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin; NMR, nuclear magnetic resonance.

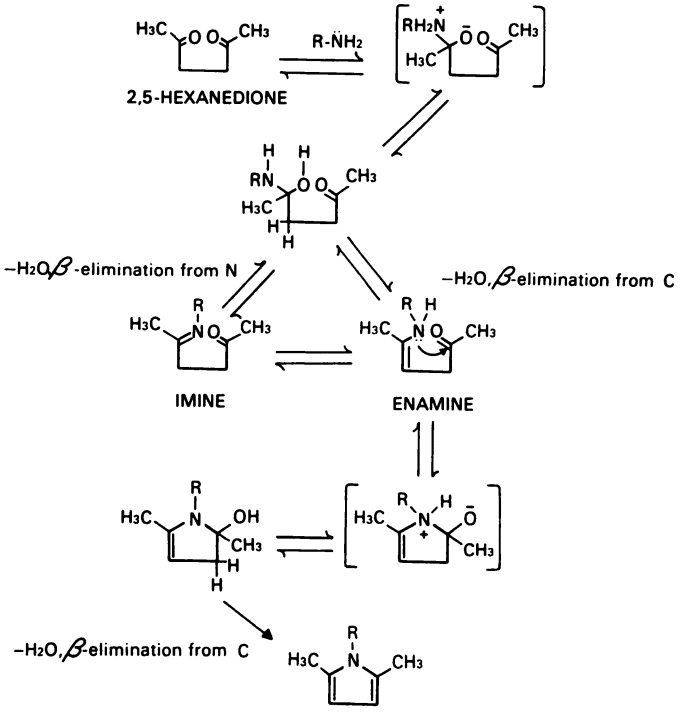

Fig. 1. Proposed mechanism of the Paal-Knorr synthesis of dialkylpyrroles from γ-diketones and primary amines (adapted from Refs. 7 and 8).

ably enter into alternative, nonpyrrole-forming reactions, such as diimine cross-linking.

Other quantitative aspects of γ-diketone-protein interaction are also unclear. The formation of stable nonpyrrole products has not been ruled out, particularly at high diketone:amine molar ratios, since other nucleophilic amino acid side chains (e.g., -SH) might also react with the carbonyl functions of γ-diketones. Early studies in this laboratory suggested that total stable covalent binding of 2,5-HD with protein as measured by radiolabeling was generally higher (on a mol/mol protein basis) than pyrrole adduct concentrations as measured with Ehrlich's reagent (DMAB) (11). This finding could be explained by two mechanisms: 1) formation of nonpyrrole reaction products or 2) inability of the DMAB assay to detect all adducts in pyrrolylated protein. The latter effect could be a reflection of protein conformational changes accompanying pyrrolylation. In view of the widespread use of the DMAB assay and the potential importance of both stable nonpyrrole products and protein conformational alterations in the mechanism of γ-diketone neuropathy (12), it was of interest to further examine this phenomenon.

The present report describes evidence for an imine intermediate during *in vitro* pyrrolylation of BSA by 2,5-HD in aqueous buffer, which supports previously proposed mechanisms for this reaction (5, 9). Quantitative comparisons are also made between total diketone binding and pyrrole adduct formation as a function of both pH and diketone:amine molar ratio. Findings are consistent with the appearance of nonpyrrole reaction products at high diketone:amine molar excess but not at lower, more physiologically relevant, ratios. The sensitivity and effectiveness of the DMAB assay in the detection of pyrrole adducts is also confirmed. Finally, the possible significance of these *in vitro* findings is discussed in relation to the proposed mechanisms of action of the neurotoxic γ-diketones.

Materials and Methods

Chemicals. 2,5-HD (Eastman Kodak, Rochester, NY) was >98% pure and was redistilled before use. [1,6-^{14}C]-2,5-HD (New England Nuclear, Boston, MA), with a specific activity of 8.77 mCi/mmol, was diluted with unlabeled 2,5-HD for covalent binding studies. BSA (globulin-free), Con A, TPCK-trypsin, and DMAB were obtained from Sigma Chemical, St. Louis, MO. Other materials included pronase (Calbiochem Chemical, San Diego, CA), proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN), $NaCNBH_3$ (Alfa Chemicals, Danvers, MA), 2,5-dimethylpyrrole (Aldrich Chemical, Milwaukee, WI), and PITC (Pierce Chemical, Rockford, IL). All chemicals were of reagent quality or better.

Incubation conditions. All 2,5-HD:protein incubations were performed in the dark with exclusion of air to eliminate secondary autoxidative modification of the pyrrole adduct (13). For this purpose, a purging system based on alternate exposure of samples to vacuum (20 mmHg) and ultra high purity N_2 was employed. Six vacuum/N_2 cycles were theoretically sufficient to reduce the O_2 partial pressure of the system to 5.55×10^{-5} mmHg (14). All exhaustive dialyses were performed in the dark under similar conditions of O_2 exclusion at 4°.

Pyrrole assay. The colorimetric assay for 2,5-dimethylpyrrole adducts was based on reaction with DMAB in the presence of an acidic catalyst and utilized a modification of a previously described method (4). Samples (0–100 μl) of pyrrolylated protein in aqueous solution were diluted to 100 μl with distilled H_2O followed by the addition of 1.3 ml of 1% (wt/vol) SDS in H_2O and 100 μl of a reagent consisting of 1.5% (wt/vol) DMAB and 1% HCl (vol/vol) in methanol:H_2O (50:50). After 10 min at room temperature, the absorbance was determined at 530 nm and compared with standard curves generated using 2,5-dimethylpyrrole. Pyrrole adduct levels were expressed either as nanomoles 2,5-dimethylpyrrole/milligram protein or as moles 2,5-dimethylpyrrole/mole protein. For assays using pronase pretreatment, samples (0–50 μl) were initially incubated (37°, 30 min, N_2) with an equal volume of a freshly prepared solution of 0.7 mg pronase/ml of 200 mM sodium phosphate buffer, pH 7.4. The total volume of the sample was then adjusted to 100 μl with H_2O and the assay continued as described above.

Effect of enzyme pretreatment on DMAB-detectable pyrrole. To determine the effects of various proteases on DMAB-detectable pyrrole, BSA (5 mg/ml in 200 mM sodium phosphate buffer with 0.1% NaN_3, pH 7.4) was incubated (37°) with a 50-fold molar excess (relative to lysine) of 2,5-HD. After 24 hr, the resulting pyrrole-BSA was exhaustively dialyzed versus 10 mM sodium phosphate buffer (pH 7.4) to remove unreacted diketone. Samples of dialyzed pyrrole-BSA were reincubated with either no enzyme or with aliquots of TPCK-trypsin, proteinase-K, or pronase in dialysis buffer (enzyme:protein weight ratio of 1:60 for all enzymes). DMAB-detectable pyrrole concentrations were determined at timed intervals as described above and after the addition of a fresh aliquot of enzyme solution.

Pyrrole formation versus [^{14}C]-2,5-HD binding. To compare DMAB-detectable pyrrole adduct levels with total covalent binding at various 2,5-HD:lysine molar ratios and as a function of pH, BSA solutions (5 mg/ml in 200 mM sodium phosphate buffer containing 0.1% NaN_3, pH 7.4 or 9.5) were mixed with aliquots of [1,6-^{14}C]-2,5-HD to yield diketone:amine molar ratios ranging from 0.2:1 to 50:1 (0.8–210 mM 2,5-HD final concentration). Incubation was continued for 24 hr at 37° in the dark under N_2. A portion of each sample was then assayed (with and without pronase pretreatment) for pyrrole content as described above. The remaining sample was exhaustively dialyzed versus 10 mM sodium phosphate buffer (pH 7.4 or 9.5), and 0.5 ml of the dialysate was added to 10 ml scintillation counting fluid (Beckman Ready-Solve, Beckman Instrument, Fullerton, CA) and counted using a Packard Tri-Carb liquid scintillation counter. Results

were expressed as moles pyrrole adduct per mole BSA or as moles bound 2,5-HD per mole BSA.

Amino acid analysis. Quantitative amino acid analysis was performed using a Waters (Milford, MA) Pico-Tag amino acid analysis system based on reverse phase high performance liquid chromatographic separation of PITC-derivatized amino acids. BSA (5 mg/ml in 200 mM sodium phosphate buffer, pH 7.4 or 9.5) was incubated with either a 5-, 50-, or 500-fold molar excess of 2,5-HD (relative to lysine) for 24 hr, followed by exhaustive dialysis in H_2O. Samples of untreated BSA in distilled water were also prepared as controls. Hydrolysis of pyrrolylated and control BSA was performed using 6 N HCl containing 1% phenol under vacuum for 24 hr at 110°, followed by PITC derivatization. Samples corresponding to 0.5 μg original protein were injected and analyzed as described above.

Effects of SDS and DTT on DMAB-detectable pyrrole adduct levels. BSA and Con A (5 mg/ml in 200 mM sodium phosphate buffer with 0.1% NaN_3, pH 7.4) were treated with a 50-fold or 100-fold molar excess (relative to lysine), respectively, of unlabeled 2,5-HD for 24 hr, followed by exhaustive dialysis. To 1-ml aliquots of treated protein was added either 250 μl H_2O, 250 μl of 10% SDS in H_2O, 250 μl 10% SDS + 5 mg DTT, or 0.6 g urea, respectively. Samples were incubated (37°C, N_2) for 2 hr, followed by the addition of 20 μl of proteinase-K solution (1 mg/ml in 10 mM sodium phosphate buffer, pH 7.4) and an additional 2-hr incubation. Pyrrole adduct levels were determined by means of DMAB assay of the original dialysate and of all samples after each incubation.

Detection and quantitation of an imine intermediate during pyrrolylation of BSA. Quantitation of imine intermediates in γ-diketone-treated protein was accomplished by determining values for covalently bound radiolabel in samples of [^{14}C]-2,5-HD-treated BSA with and without treatment with $NaCNBH_3$ before exhaustive dialysis. This reagent reduces labile imines to stable secondary amines without affecting carbonyl or other unsaturated functions (15). BSA (7.5 mg/ml in 200 mM sodium phosphate with 0.1% NaN_3, pH 7.4 or 9.5) was incubated with a 2-fold molar excess (relative to lysine) of [1,6-^{14}C]-2,5-HD for times ranging from 2 to 144 hr. Incubated samples were then divided into equal portions, one of which was treated (20 min, 37°, N_2) with $NaCNBH_3$ at a 50-fold molar excess (relative to lysine). All samples were then exhaustively dialyzed versus 10 mM sodium phosphate buffer (pH 7.4 or 9.5) to remove unreacted 2,5-HD and $NaCNBH_3$. Total, covalently bound radioactivity was determined by liquid scintillation counting of dialyzed samples. This methodology yielded a measure of imine formation as the difference in total [^{14}C]-2,5-HD binding between $NaCNBH_3$-treated and untreated samples. To confirm the lack of effect of $NaCNBH_3$ on already formed adducts, pyrrole adduct concentrations were also determined in all samples using the previously described DMAB assay.

Results

Effect of proteolytic enzymes on DMAB-detectable pyrrole in pyrrole-BSA. Preincubation of pyrrole-BSA with TPCK-trypsin, pronase, or proteinase-K for various periods before the DMAB-based pyrrole assay was tested as a means of enhancing the sensitivity of the assay. All three enzymes produced increases in DMAB-detectable pyrrole levels, although the increases seen with the nonspecific enzymes pronase and proteinase-K were markedly higher than those with TPCK-trypsin. After 15-min incubation with TPCK-trypsin, pronase, or proteinase-K, the amount of DMAB-detectable pyrrole was increased to 115, 165, and 170%, respectively, of that measured in untreated pyrrole-BSA. A 2-hr preincubation resulted in only slight additional increases in pyrrole levels. Addition of a fresh aliquot of enzyme at 2 hr did not result in further increases in DMAB-detectable pyrrole. Proteinase-K was also found to be active in the presence of 8 M urea, 2% SDS, and 20 mM DTT.

Effects of SDS, urea, and DTT on DMAB-detectable pyrrole levels in pyrrole-BSA. Additional attempts were made to maximize DMAB-detectable pyrrole concentrations by the preincubation of pyrrole-BSA with protein denaturants (SDS, urea) with or without a thiol reducing agent (DTT). Results of these treatments are shown in Fig. 2A. Pretreatment with 2% SDS alone resulted in a 45% increase in DMAB-detectable pyrrole, whereas exposure to both 2% SDS and 20 mM DTT produced a 35% decrease in detectable pyrrole com-

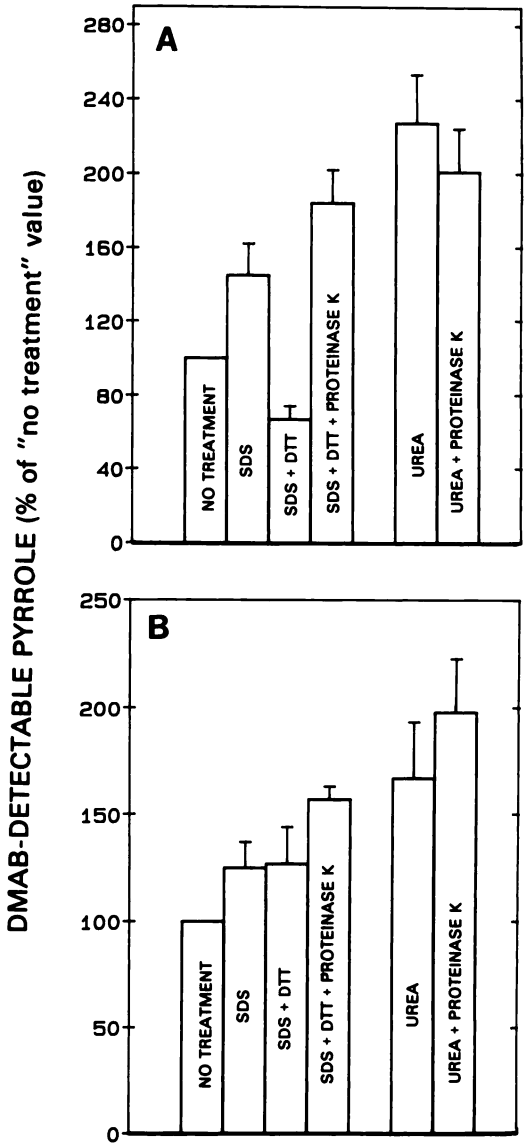

Fig. 2. Effects of denaturants, a thiol-reducing agent, and protease pretreatment on DMAB-detectable pyrrole concentration in pyrrole-BSA and pyrrole-Con A. Samples of pyrrole-BSA (A) or pyrrole-Con A (B) (prepared as described under Materials and Methods) were sequentially treated with either SDS, DTT, and proteinase-K or with urea and proteinase-K. DMAB-detectable pyrrole was assayed after each treatment step and compared with untreated samples. To control for variations in the DMAB-detectable pyrrole content of untreated pyrrole-BSA between experiments, data are expressed as the percentage of the untreated value (assigned 100%). Data represent mean and standard deviation of triplicate determinations from three separate experiments. Note the decrease in DMAB-detectable pyrrole concentrations after SDS + DTT treatment in pyrrole-BSA but not in pyrrole-Con A.

pared with untreated pyrrole-BSA. Subsequent treatment of SDS- and DTT-treated pyrrole-BSA with proteinase-K resulted in an 85% increase in pyrrole levels compared with the original sample. Incubation with 8 mM urea was somewhat more effective than 2% SDS in raising detectable pyrrole levels (Fig. 2A).

To explore the possibility that the decreased DMAB-detectable pyrrole in pyrrole-BSA treated with DTT was related to disulfide cleavage, similar studies were performed with Con A, a protein that lacks cysteine sulfhydryl moieties (16). In contrast to pyrrole-BSA, both SDS and SDS + DTT pretreatment produced a slight (25%) increase in DMAB-detectable pyrrole in pyrrole-Con A (Fig. 2B). As with pyrrole-BSA, additional enzyme treatment further increased detectable pyrrole levels, and 8 M urea was more effective than 2% SDS as an enhancer of DMAB-detectable pyrrole (Fig. 2B). No combination of denaturant and enzyme was effective in raising DMAB-detectable pyrrole levels significantly more than 2-fold over the original sample with either pyrrole-BSA or pyrrole-Con A.

Pyrrole formation versus total 2,5-HD binding. To evaluate the relationships between DMAB-detectable pyrrole, total covalent diketone binding, incubation pH, and the 2,5-HD:lysine molar ratio, additional *in vitro* BSA incubation studies were performed using [1,6-^{14}C]-2,5-HD. The DMAB assay was performed on pyrrole-BSA with or without pronase pretreatment.

The relationship between DMAB-detectable pyrrole adduct levels (moles 2,5-dimethylpyrrole/mole protein) and total binding (mol [^{14}C]-2,5-HD/mol protein) was found to be dependent on both the diketone concentration and the incubation pH (Table 1). Correspondence between pyrrole concentrations and total binding levels was seen only at the lowest 2,5-HD:lysine molar ratio of 0.2:1, at either pH. At higher molar ratios, total covalent binding was consistently greater than DMAB-detectable pyrrole concentrations, and this divergence was more pronounced at pH 7.4 than at pH 9.5 (Table 1, final column).

TABLE 1

Total stable covalent binding of 2,5-HD versus DMAB-detectable pyrrole in 2,5-HD-treated BSA

2,5-HD:Lysine molar ratio	Pyrrole concentration[a]		Total covalent binding[b]	Relative concentration[d]
	−Pronase	+Pronase		
	mol pyrrole/mol protein		*mol [^{14}C]-2,5-HD/mol protein*	
pH 7.4[c]				
50:1	3.9 ± 0.3	6.6 ± 0.6	20.1 ± 1.9[e]	0.33
5:1	1.0 ± 0.1	1.0 ± 0.1	2.3 ± 0.3[e]	0.44
1:1	0.34 ± 0.02	0.32 ± 0.02	0.52 ± 0.02[e]	0.65
0.2:1	0.024 ± 0.001	ND	0.020 ± 0.001	1.20
pH 9.5[c]				
50:1	5.5 ± 0.1	17.2 ± 2.3	32.6 ± 2.0[f]	0.53
5:1	2.7 ± 0.1	4.3 ± 0.7	5.8 ± 0.6[g]	0.74
1:1	1.5 ± 0.3	2.1 ± 0.6	2.0 ± 0.5	1.05
0.2:1	0.18 ± 0.08	ND	0.20 ± 0.06	0.90

[a] Determined by DMAB assay with or without pronase pretreatment as discussed in Materials and Methods. Data are means ± SD of triplicate samples from three separate experiments; ND, not determined.

[b] Determined by liquid scintillation counting of [^{14}C]-2,5-HD-treated BSA after exhaustive dialysis.

[c] Incubation performed under N_2 (37°, 24 hr) in 200 mM sodium phosphate buffer with 0.1% NaN_3 at given pH.

[d] Ratio of values for pyrrole concentration/total covalent binding. Larger value of +/− pronase data used for numerator.

[e] Significantly different (Student's *t* test) from pyrrole concentration value (larger value of −/+ pronase data used), $p \leq 0.001$.

[f] $p \leq 0.01$.

[g] $p \leq 0.05$.

Attempts to further reduce measured levels of bound 2,5-HD by exhaustive dialysis versus 10% SDS or 8 M urea or by extraction with organic solvents did not yield data significantly different from those obtained by dialysis versus buffer alone. Values for both total binding and pyrrole concentration were generally higher at pH 9.5 than at pH 7.4 at all molar ratios tested.

Pronase pretreatment substantially increased the amount of DMAB-detectable pyrrole at the higher diketone:lysine molar ratios (50:1 for pH 7.4; 50:1, 5:1, and 1:1 for pH 9.5) (Table 1). The absolute increase was more pronounced at pH 9.5 than pH 7.4 (i.e. 3.1-fold versus 1.7-fold greater pyrrole levels compared with untreated pyrrole-BSA at 50:1). Enzyme treatment was effective in producing DMAB-detectable pyrrole levels equivalent to diketone binding values in the 1:1 molar ratio (pH 9.5) samples. In contrast, pronase pretreatment had no effect on DMAB-detectable levels in the 1:1 samples at pH 7.4 (Table 1).

Amino acid analysis. Results of quantitative amino acid analysis of BSA treated (24 hr, 37°) with 2,5-HD at 5:1, 50:1, or 500:1 diketone: lysine molar excess at either pH 7.4 or 9.5 are shown in Table 2. Significant decreases in levels of lysine were noted at both the 50:1 and the 500:1 ratios, whereas histidine content was slightly lower at 500:1. No alterations in the levels of any other amino acid were observed, indicating the absence of any other acid-stable adducts. The decreases in lysine were more pronounced at pH 9.5 than pH 7.4, although complete lysine conversion was not seen even under conditions of extreme diketone excess. At the 50:1 molar ratio, values for loss of lysine (moles/mole protein) correlated well with those for pyrrole formation determined by DMAB assay after pronase pretreatment (Table 1) (i.e., 5.0 mol lysine converted versus 6.6 mol pyrrole formed/mol protein at pH 7.4 and 14.6 mol lysine converted versus 17.2 mol pyrrole formed/mol protein at pH 9.5). No new amino acid peak corresponding to the lysine-2,5-HD reaction product (i.e., ϵ-*N*-[2,5-dimethylpyrrolyl]norleucine) was observed, although an increase in late-eluting, polymeric material was noted in heavily pyrrolylated samples. This

TABLE 2

Quantitative amino acid analysis of 2,5-hexanedione-treated BSA[a]

2,5-HD:Lysine incubation ratio	Amino acid content	
	pH 7.4	pH 9.5
	mol/mol protein	
Lysine		
Control[b]	60.3 ± 2.9	60.3 ± 2.9
5:1	57.6 ± 4.3	57.4 ± 1.8
50:1	55.3 ± 1.2[c]	45.7 ± 1.8[d]
500:1	31.7 ± 4.0[d]	19.8 ± 1.1[d]
Histidine		
Control[b]	16.9 ± 0.5	16.9 ± 0.5
5:1	17.0 ± 0.7	16.6 ± 0.8
50:1	16.6 ± 0.8	15.7 ± 1.0
500:1	15.1 ± 1.1[c]	14.7 ± 0.7[e]

[a] Samples of BSA [5 mg/ml in 200 mM sodium phosphate buffer (pH 7.4) or 9.5] were incubated (37°, N_2, 24 hr) with 2,5-HD at diketone:lysine molar ratios of 5:1, 50:1, or 500:1 as indicated, followed by exhaustive dialysis with H_2O. Aliquots of dialysate were hydrolyzed in 6 N HCl with 1% phenol, derivatized with PITC, and analyzed by reverse-phase high performance liquid chromatography as discussed under Materials and Methods. Tryptophan was not determined. Data are means ± SD of quadruplicate determinations.

[b] Untreated BSA in distilled water.

[c] Significantly different (Student's *t* test) from control, $p \leq 0.05$.

[d] $p \leq 0.001$.

[e] $p \leq 0.01$.

result is consistent with the previously demonstrated acid sensitivity of the derivative (4).

Detection of an imine intermediate during pyrrolylation of BSA. Results of studies comparing covalent binding of [^{14}C]-2,5-HD to BSA with and without $NaCNBH_3$ treatment are shown in Fig. 3. A significantly higher level of binding was noted with the $NaCNBH_3$-treated compared with the untreated samples at incubation times of 2–24 hr. This difference in binding, which represents imine intermediate, was approximately constant during this time, a finding consistent with steady-state production and reaction of the imine. Relative levels of imine were approximately 4-fold higher at pH 9.5 than at pH 7.4, as were absolute levels of total [^{14}C]-2,5-HD binding. In contrast, no significant difference between $NaCNBH_3$-treated and untreated samples was apparent after 144 hr of [^{14}C]-2,5-HD:BSA incubation, although the absolute levels of binding were substantially higher than at 24 hr. No differences in DMAB-detectable pyrrole were observed between $NaCNBH_3$-treated and untreated samples at any time, indicating that the $NaCNBH_3$ treatment had no effect on already formed pyrrole adducts (data not shown).

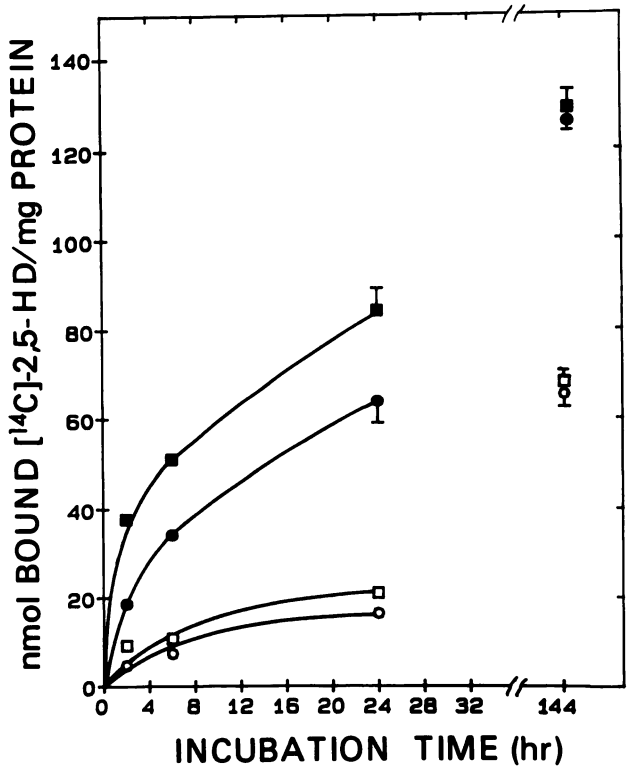

Fig. 3. Quantitation of imine formation in 2,5-HD:BSA incubation mixtures as a function of time and incubation pH. Samples of BSA in buffer were incubated (37°C, N_2) with [^{14}C]-2,5-HD at a 2:1 diketone:amine molar ratio at pH 7.4 (*open symbols*) or pH 9.5 (*closed symbols*) for 2–144 hr. Aliquots of samples were withdrawn and either immediately dialyzed (○,●), or treated with $NaCNBH_3$ (see under Materials and Methods) and then dialyzed (□,■). Dialyzed samples were subjected to liquid scintillation counting to determine total stable 2,5-HD binding. The difference in total binding between $NaCNBH_3$-treated and untreated samples represents imine intermediate. Data represent mean and standard deviation of triplicate determinations from three separate experiments. If not indicated, the error bar lies within the symbol. Values for $NaCNBH_3$-treated samples are significantly different (Student's *t* test; $p \leq 0.01$); from values for corresponding untreated samples at all incubation times except 144 hr.

Discussion

Pyrrole adduct formation in NF protein has been implicated as a critical step in the mechanism of induction of neuropathy by the γ-diketones (4). Although pyrrolylation of protein has been demonstrated in a number of *in vitro* and *in vivo* systems (4–6), several aspects of this reaction remain unclear. These uncertainties involve the identification of the rate-limiting steps and intermediates in the reaction, the possible formation of nonpyrrole products, and the effects of pyrrolylation on protein conformation. Figure 4 summarizes the demonstrated and hypothetical reaction pathways of the γ-diketones with protein.

Early investigators proposed imines as intermediates in the Paal-Knorr synthesis under organic reaction conditions (7, 8). The imine was postulated to undergo tautomerization to the enamine, followed by cyclization to the pyrrole. However, these workers did not present conclusive evidence in support of such a mechanism. Recently, Katritzky and coworkers (9), using ^{1}H-, ^{13}C-, and ^{15}N-NMR spectroscopy, provided data in support of an N-substituted imine intermediate in the reaction under nonaqueous conditions. Two rate-limiting steps were identified; the initial nucleophilic attack of amine nitrogen on carbonyl carbon and the subsequent attack of enamine nitrogen on the second carbonyl group. The enamine itself was not detected, presumably due to the unfavorable equilibrium for its formation (Fig. 4).

The present investigation provides evidence for a similar mechanism for pyrrolylation of protein by 2,5-HD under aqueous conditions. At low diketone:amine molar ratios, a steady-state level of imine was rapidly achieved, accompanied by progressive formation of stable product. Such findings are consistent with a primary rate-determining step of intramolecular attack of the enamine on the second carbonyl function of 2,5-HD. With prolonged incubation, there appeared to be a shift in the rate-determining step to the initial amine-carbonyl attack, since the imine intermediate was no longer detectable, despite the presence of both unreacted 2,5-HD and lysine ε-amine moieties. This finding is significant since it suggests that only a limited number of the lysines in BSA are susceptible to reaction with 2,5-HD under these conditions. The marked enhancement of both imine and pyrrole formation at the higher pH is not surprising and probably reflects the lesser degree of protonation of the reacting amine.

The demonstration of appreciable levels of imine intermediate in γ-diketone-protein mixtures raises two major considerations. First, these derivatives may be vulnerable to attack by another protein ε-amine moiety via the second unreacted carbonyl function. This would yield a diimine, resulting in formation of an intermolecular or intramolecular crosslink (Fig. 4). Such derivatives (and their hypothetical stable conjugated diimine oxidation products) were originally proposed to explain the observed aggregation of neurofilaments in γ-diketone neuropathy (17). However, diimines would be relatively labile at physiological pH, and no evidence for their presence in γ-diketone-treated protein is available. NF aggregation has since been attributed to autoxidative reactions involving pyrrole adducts (5, 6), probably yielding stable pyrrole-pyrrole crosslinks (13) (Fig. 4). Second, since the rate-limiting step involves C-H bondbreaking (Fig. 1), a primary isotope effect should be apparent in this reaction. We have recently confirmed such an effect using perdeuterio-2,5-HD, a derivative that formed the

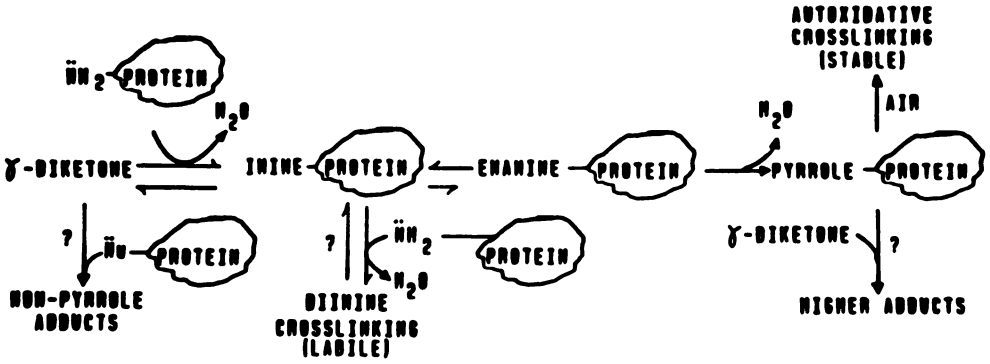

Fig. 4. Demonstrated and hypothetical reaction pathways between γ-diketones and protein. Ñu, protein nucleophile other than the amino group (e.g., -SH). *Question mark*, proposed alternative pathway.

pyrrole at a slower rate and that was significantly less neurotoxic than native 2,5-HD in the rat (18).

Evidence from the present investigation also supports the formation of stable nonpyrrole reaction products at high γ-diketone: amine molar ratios. Levels of total stable [^{14}C]-2,5-HD binding were consistently greater than DMAB-detectable pyrrole adduct concentrations at these ratios. Despite this discrepancy, the efficiency of the DMAB assay was verified by the high correspondence between values for pyrrole concentrations as measured by DMAB and for lysine conversion as determined by quantitative amino acid analysis. Thus the excess in stable [^{14}C]-2,5-HD binding was not simply a reflection of insensitivity of the DMAB assay. This is an important finding in view of the wide use of this assay among investigators examining mechanisms of γ-diketone neuropathy.

One mechanism that could explain extra nonpyrrole binding in 2,5-HD-treated protein would invoke covalent reaction of the diketone with nucleophilic amino acid side chains other than lysine ε-amine (Fig. 4). For example, as has been suggested (19), reaction of γ-diketones with protein sulfhydryl moieties might occur, a scheme that would yield hemithioketals. However, since BSA has only one free sulfhydryl group (20), hemithioketal formation could only explain a small fraction of the observed nonpyrrole binding in the present study. Reaction with the amide nitrogens of asparagine and glutamine could result in products not detectable by amino acid analysis, because these would be hydrolyzed to aspartic and glutamic acid, respectively, under the acidic conditions employed. However, previous studies utilizing either alkaline or total enzymatic hydrolysis techniques failed to detect significant reactivity of these amino acids (4).

Since quantitative amino acid analysis revealed a decrease only in the peak corresponding to lysine in 2,5-HD-treated BSA (except for minor histidine reactivity at extreme 2,5-HD molar excess), it is conceivable that the diketone may react with previously formed pyrrole derivatives to yield higher adducts (Fig. 4). In this case, total [^{14}C]-2,5-HD binding on a mole basis would be greater than either lysine conversion values derived from amino acid analysis or DMAB-detectable pyrrole concentrations. Although no supportive analytical data are available from the present study, 2,5-dialkyl-substituted pyrroles can readily react with aldehydes and ketones via electrophilic aromatic substitution at ring positions 3 and 4 (21). In neutral or slightly basic solution, the major products are stable pyrrole carbinols, which can dehydrate to pyrrolenines as the pH is lowered (21). In addition, formation of both isoindoles and isoindolines has been reported to occur in aqueous solutions of 1,2,5-trisubstituted pyrroles and 2,5-HD using acidic catalysts and refluxing conditions (22–24). These derivatives are relatively unstable and would be expected to undergo additional rearrangements to more complex products. Further analytical studies will be required to evaluate the likelihood of formation of these higher adducts under physiological conditions.

Conformational alterations in the NF proteins as a result of pyrrolylation have been suggested to underlie γ-diketone neuropathy (12). Thus the effects of proteases and denaturing agents on the detection of pyrrole adduct by DMAB are of considerable interest. Although TPCK-trypsin produced a slight increase in DMAB-detectable pyrrole in pyrrole-BSA, the relatively nonspecific enzymes pronase and proteinase-K were much more effective in this regard. These findings suggest that pyrrolylation may be accompanied by conformational changes resulting in decreased accessibility of the DMAB reagent to the pyrrole adduct. In view of the marked hydrophobicity of alkylpyrroles, such effects would be consistent with a tendency toward translocation of the adduct into the interior of the protein. Nonspecific protease treatment may therefore serve to expose "buried" adducts sufficiently to allow reaction with DMAB.

Further evidence for structural rearrangements in pyrrolylated protein was obtained by examining the effects of SDS, DTT, and protease on DMAB-detectable pyrrole. It was initially reasoned that treatment of pyrrole-BSA with a combination of a denaturant (SDS) and a thiol reductant (DTT) would result in complete unfolding of the protein and, consequently, complete exposure to the DMAB reagent. Instead, combined SDS and DTT exposure lowered DMAB-detectable pyrrole levels to below those seen with either untreated or with SDS-treated pyrrole-BSA, an effect that was reversible on subsequent protease treatment. In contrast, SDS and DTT treatment of pyrrole-Con A had the same effect on DMAB-detectable pyrrole as SDS treatment alone. A possible explanation for the disparate effects of DTT might be related to the release of conformational restraints accompanying disulfide-bond cleavage. In the case of pyrrole-BSA, this would allow a refolding of the protein into its lowest energy form (i.e. one in which hydrophobic residues are localized to the interior). Since pyrrole-Con A has no disulfide linkages (16), DTT treatment would be expected to have no effect.

The present study has illuminated many quantitative aspects of the Paal-Knorr reaction between γ-diketones and protein *in vitro*. Although it is premature to extend all of these findings to the *in vivo* milieu, certain generalizations may be appropriate. For example, it appears that, at the relatively low levels of

lysine conversion likely to be present after 2,5-HD exposure *in vivo* (6), the DMAB assay can accurately assess protein pyrrole adduct concentrations. In addition, evidence has been provided that pyrrolylation can result in significant alterations in protein structure, particularly in those species not constrained by multiple disulfide linkages, an effect presumably related to the extreme hydrophobicity of this derivative. We have previously proposed that the carboxy-terminal "tail" region of the NF-M and NF-H subunit proteins may represent the critical target sites for 2,5-HD binding in this neuropathy (6, 12). These regions are thought to interact with other cytoskeletal proteins (25, 26) and to lack appreciable secondary structure or disulfide stabilization (27, 28). It is reasonable to expect that pyrrolylation of NF protein in these "tail" regions could result in structural changes that are ultimately detrimental to the function of these axonal cytoskeletal elements.

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