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Imprinting of Lyophilized α-Chymotrypsin Affects the Reactivity of the Active-Site Imidazole

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Iodomethane reacted *in vacuo* with lyophilized α-chymotrypsin to give an inactive enzyme in which the active-site imidazole was dimethylated. However, α-chymotrypsin co-lyophilized with the competitive inhibitors, *N*-acetyl-L-tryptophan or *N*-acetyl-D-tryptophan, was fully protected from such inactivation. In contrast, indole by itself not only did not protect the lyophilized enzyme from inactivation by iodomethane but also increased the rate of inactivation. The lyoprotectants citrate or sorbitol also showed opposite effects when co-lyophilized with α-chymotrypsin. Citrate protected the lyophilized enzyme from inactivation, while bound sorbitol dramatically accelerated the inactivation. Imprinting of lyophilized α-chymotrypsin with indole or sorbitol increased the reactivity of the active-site histidine towards iodomethane. Co-lyophilization of α-chymotrypsin with appropriate ligands is known to increase significantly its enzymatic activity in hydrophobic organic solvents. It is proposed that this imprinting phenomenon arises because a greater proportion of the active-sites in the lyophilized enzyme are in a catalytically favorable conformation where the imidazole of His-57 is more strongly hydrogen bonded to the carboxylate of Asp-102.

The fact that lyophilized enzymes have substantial catalytic activity in anhydrous organic solvents[1-5] implies that a portion of the active-sites retain an active conformation under nonaqueous conditions. Active-site titration data[6,7] and solid-state NMR[8] studies of lyophilized chymotrypsin indicate that the active-site of the enzyme consists of a mixture of active and inactive conformations. The addition of ligands to the aqueous solution prior to lyophilization has been shown to increase the catalytic activity of the lyophilized enzyme in organic solvents[9-11]. In the case of chymotrypsin, lyophilization in the presence of competitive inhibitors had a substantial activation effect[5]. Other multifunctional ligands such as sorbitol which are structurally unrelated to substrates also have an activating effect[9,10] and appear to act as lyoprotectants maintaining the conformational integrity of the active-site. It has been postulated[11-13] that the binding or association of such ligands imprints[12, 14-17] a catalytically favorable conformation in the catalytic site of lyophilized enzymes. This hypothesis implies that the chemical properties of active-site functional groups are altered in the imprinted enzyme. In this report, we describe a novel approach using an *in vacuo* chemical modification of lyophilized proteins[18, 19] to investigate the chemical reactivity of the active-site imidazole of His-57 of α-chymotrypsin lyophilized in the presence and absence of imprinting ligands.

MATERIALS AND METHODS

Materials. Bovine α-chymotrypsin (EC 3.4.21.1) (lot # 91H7195), iodomethane, *N*-acetyl-L-tryptophan, *N*-acetyl-D-tryptophan, indole, D-sorbitol and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were purchased from Sigma Chemical Co. Sodium citrate was obtained from Fisher Scientific Co. All other reagents and chemicals were high purity preparations obtained from commercial sources.

Sample preparation. Stock solutions (20 mM) of ligands were adjusted to pH 8.0 with dilute NaOH. An aliquot (5.0 ml) of a ligand stock solution was added to 1.0 ml of α-chymotrypsin (1.0 mg/ml) in distilled water and the volume adjusted to 10.0 ml with distilled water to give a solution containing α-chymotrypsin (100 μg/ml) and ligand (10.0mM). Each α-chymotrypsin-ligand solution was adjusted to pH 8.0 with dilute base, transferred in 1.0 ml aliquots to Pyrex glass hydrolysis tubes and lyophilized. *In vacuo* reaction with iodomethane (25 μl) was carried out at 75°C as described previously[18]. At various time points the reaction was stopped by trapping the iodomethane by immersing the side arm of the reaction vessel in liquid nitrogen and releasing the vacuum. The samples were stored over P_2O_5 under vacuum until they were assayed.

Quantification of enzymatic activity. Samples were dissolved in 1.0 ml sodium formate (100mM) pH 4.0. Enzymatic activity was quantified on aliquots (100 μl) using a pH stat to determine the rate of hydrolysis of ATEE (10.0 mM) in a 5.0 ml reaction volume with 0.100 M NaOH as titrant. Solvent conditions were 20°C in 0.10 N KCl and 5% (v/v) acetonitrile.

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TABLE 1

Catalytic Activity of Lyophilized α-Chymotrypsin after Reaction with Iodomethane in the Absence and Presence of Ligands

	Relative activity[a]								
	Control Time h					Reaction with Iodomethane Time h			
Ligand	0	2	4	8	12	2	4	8	12
None	1.0	1.0	0.99	1.1	0.99	0.63	0.54	0.41	0.33
N-Ac-L-Trp	1.1	1.1	1.2	1.2	1.0	1.1	1.1	0.98	0.99
N-Ac-D-Trp	1.1	1.1	1.0	0.97	0.93	1.0	1.1	1.0	0.95
Indole	0.90	0.91	1.0	0.97	0.93	0.36	0.33	0.22	0.08
Citrate	1.0	0.99	0.97	1.0	1.0	0.98	1.0	1.0	0.99
Sorbitol	0.99	0.94	0.98	0.87	0.90	0.13	0	0	0

[a] The activities are relative to unheated α-chymotrypsin lyophilized at pH 8.0. The values reported are the average of two independent experiments. Mean deviations in all cases were less than 10%.

RESULTS AND DISCUSSION

Lyophilized α-chymotrypsin (LpH 8.0) retained full activity after heating *in vacuo* for 12 h at 75°C. Under the same conditions, the ligand-free lyophilized enzyme lost most of its catalytic activity after methylation for 12h with iodomethane (Table 1). Lyophilization in the presence of ligands did not alter the thermal stability of the enzyme, however the ligands did have significant effects on the inactivation by iodomethane. The L and D isomers of *N*-acetyltryptophan effectively protected the lyophilized enzyme from inactivation. Indole did not protect, but surprisingly accelerated the rate of inactivation. Citrate and sorbitol which have no structural similarity to any substrate of chymotrypsin had strikingly distinct but opposite effects. Citrate effectively prevented inactivation whereas sorbitol dramatically accelerated the inactivation.

The histidine residues of lyophilized α-chymotrypsin are dimethylated in octane or *in vacuo* at LpH 8.0[18]. Chemical analysis and ^{13}C NMR spectra after reaction of proteins with [^{13}C]iodomethane for 24 h at 75°C shows complete reaction with imidazole side-chains and less than 20% reaction with amino groups[18]. Acetylation of the ϵ-amino groups does not affect the enzymatic activity of chymotrypsin[20], and methylation, which retains the charge on the amino groups, is therefore even less likely to affect activity. The observation that full activity is retained after methylation in the presence of citrate or *N*-acetyltryptophan is consistent with modification of amino groups not affecting the catalytic activity. It therefore appears that the inactivation of the enzyme is entirely due to dimethylation of the imidazole side-chain of the active-site histidine. Further evidence for this was obtained by differential labeling in which chymotrypsin co-lyophilized with *N*-acetyl-L-tryptophan (10 mM) was reacted with [^{12}C]-iodomethane at LpH 8.0 for 48 h, followed by dialysis in water to remove the ligand. Subsequent reaction of the ligand-free ^{12}C-methylated lyophilized protein with [^{13}C]iodomethane gave the characteristic ^{13}C-resonances at 34.08 and 36.59 ppm for dimethylhistidine[18] indicating that the imidazole of His-57 was protected by the bound ligand.

The increased rate of inactivation of lyophilized α-chymotrypsin with bound indole was unexpected. This must be due to the increased rate of methylation of a catalytically essential functional group. The NMR evidence obtained in the present study and in previous studies[18, 19] shows that the only group in the active-site that is methylated by iodomethane is the imidazole of His-57. From the data in Table 1, it appears that the rate of inactivation of the ligand-free lyophilized chymotrypsin is faster in the early stages of the reaction. This observation can be explained by the existence of two populations of the enzyme active-site in the lyophilized enzyme (Fig. 1) in which iodomethane reacts more rapidly with one population. After 2h, the data fit a first order inactivation process (Fig. 2), but clearly in the initial stages the reaction is occurring by a more rapid process. Extrapolation of the linear region to zero time gives an intersection of -0.35 on the y-axis, which corresponds to 30% of the active sites in the lyophilized enzyme being present in the most reactive form. These observations are consistent with active-site titrations[6, 7] which indicate that the active-sites of lyophilized enzymes are a mixture of active and inactive conformations.

The crystal structure of α-chymotrypsin indicates the active-site groups of Asp-102, His-57 and Ser-195 are in position to form a hydrogen-bonded triad[21]. However, the crystal structure of γ-chymotrypsin indicates that this hydrogen-bonded system is altered at moderately alkaline pH values[22] used in the present study. Nevertheless, it is generally agreed that hydrogen bonding of the imidazole side-chain of His-57 with the

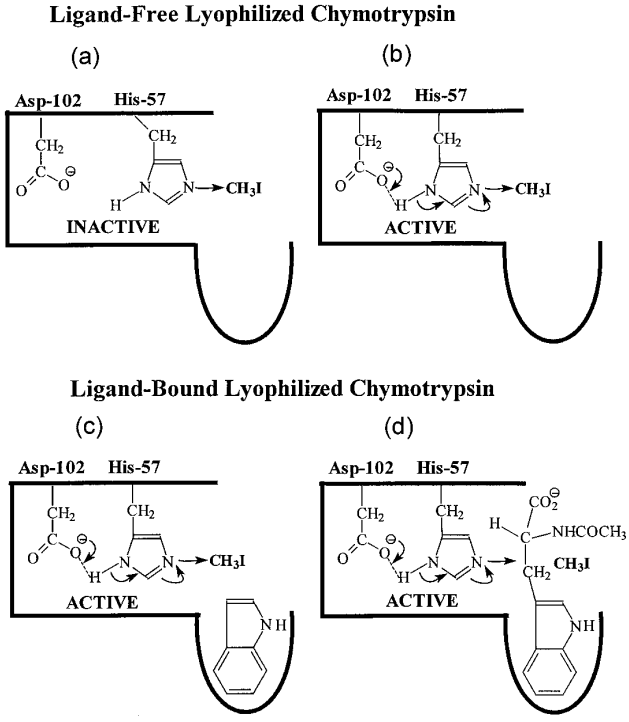

FIG. 1. Schematic representation of the effect of ligand binding on the reactivity of the imidazole moiety of His-57 in lyophilized α-chymotrypsin. The open ellipsoid structure depicts the hydrophobic binding pocket. The side-chains of Asp-102 and His-57 are positioned in proximity but only interact in the active conformations of the lyophilized enzyme. Iodomethane reacts with all conformations except (d) where the active-site functional groups are shielded by the bound ligand. Conformations (b) and (c) react more rapidly than (a) due to the enhanced nucleophilicity of the imidazole moiety.

side-chains Asp-102 and Ser-195 is a crucial component of the catalytic process[23-25]. The fact that the imidazole of His-57 reacts so readily with iodomethane provides further evidence that Ser-195 does not form a strong hydrogen bond with it in the absence of bound substrate[26]. It therefore seems reasonable to propose that the catalytic triad of α-chymotrypsin lyophilized at pH 8.0 will consist of two or more hydrogen-bonded forms of the imidazole of His-57 with the carboxylate of Asp-102. Due to the conformational rigidity of the lyophilized protein[27-30], conformations such as that depicted in Fig. 1a where the imidazole of His-57 is not positioned to interact optimally with carboxylate Asp-102 would be catalytically less active or inactive. When lyophilized in the presence of a ligand which binds in the active-site, a greater proportion of the active-sites in the lyophilized enzyme are maintained in a catalytically active conformation (Fig. 1b, 1c) where the imidazole moiety is optimally hydrogen-bonded to the carboxylate of Asp-102. Such an interaction would be expected to increase the nucleophilicity of the imidazole group and therefore increase the rate of reaction with electrophilic reagents such as iodomethane. In fact, the active-site imidazole of His-57 of aqueous chymotrypsin[31] and chymotrypsinogen[32] have been found to be super-reactive toward the electrophilic reagent 1-fluoro-2,4-dinitrobenzene.

The present results provide further evidence for the existence of an imprinting phenomenon which affects the structure of the active-site and are consistent with hydrogen bonding between His- 57 and Asp-102 as part of this process. It is possible that *N*-acetyltryptophan also induces the formation of a hydrogen bond of the active-site imidazole with the hydroxyl of Ser-195, as has been proposed for substrates[26], thereby preventing reaction with iodomethane. The other possibility is that the amino acid functionality simply shields the catalytic groups and prevents reaction with iodomethane as shown in Fig. 1d. Whichever explanation is correct, it is clear that *N*-acetyltryptophan imprints the lyophilized enzyme by affecting the hydrogen bonding of the catalytic triad.

The reason citrate prevented and sorbitol accelerated the inactivation is not obvious. Both are multifunctional molecules that are believed to interact with the protein primarily through hydrogen bonding during the lyophilization process to minimize protein-protein contacts[13, 14, 33]. The present data indicates that the association of citrate with the lyophilized enzyme shields the catalytic site and prevents methylation of His-57. In contrast, it appears that sorbitol does not shield the active-site but does interact with other regions of the molecule in such a manner as to maintain the active-site of the lyophilized enzyme in a catalytically active conformation. Like indole, it does not prevent methylation of His-57, but it imprints α-chymotrypsin with a higher proportion of the active-sites in an active conformation (Fig. 1b), and this is reflected in a more rapid inactivation by iodomethane. Sorbitol has many interaction sites with the protein that provide a much greater stabilization of the native structure and the active-site in the lyophilized state.

A significant difference between lyophilized protein

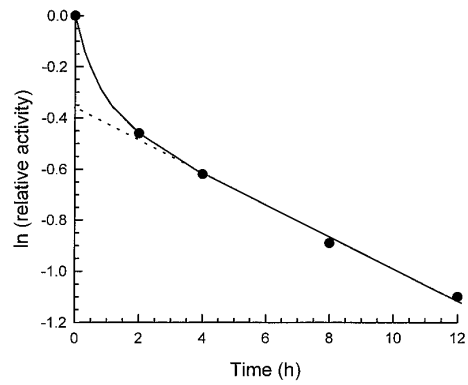

FIG. 2. First order plot for the inactivation of lyophilized α-chymotrypsin by reaction with iodomethane.

and protein in solution is that there are no dynamic conformational or protonic equilibria in the lyophilized state. This gives rise to two phenomena. One is pH memory[5, 27-28] in which the protein "remembers" the pH of the solution from which it was prepared, i.e. the ionizable functional groups have the same degree of ionization as in the aqueous solution but there is no exchange of protons. The other is molecular memory[12, 14-17] in which the protein "remembers" the conformation imprinted by a ligand co-lyophilized with the protein, after removal of the ligand. In the present study, we have taken advantage of these phenomena to carry out a chemical reaction on chymotrypsin that could not be performed in aqueous solution. Preparing the lyophilized protein at LpH 8.0 greatly increased the selectivity towards modification of the imidazole groups since the majority of the amino groups were protonated and therefore unreactive towards iodomethane. In aqueous media, the L and D enantiomers of *N*-acetyltryptophan are relatively weakly bound to chymotrypsin[34] with association constants of the order of 10^2. In solution, they would offer little protection to chemical modification as a significant portion of the free enzyme would be present in equilibrium with the enzyme-inhibitor complex and, due to Le Châtelier's principle, the active-sites would become completely modified.

The effect of indole and sorbitol in increasing the apparent rate of methylation of the active-site histidine indicates that the imprinting phenomena are involved in orienting the catalytic triad of chymotrypsin for optimal catalytic activity. Lyophilization in the presence of some inorganic salts have also been reported to dramatically increase the activity of enzymes in organic solvents[35], and presumably, they affect the conformation of the active-site when co-lyophilized with the enzyme. We have also found in preliminary investigations that the presence of various inorganic anions and cations alters the rate of reaction of iodomethane with functional groups in lyophilized α-chymotrypsin and other proteins, however their effects appear to be more complex than with organic ligands. In the presence of 5 mM phosphate, for example, *N*-acetyl-L-tryptophan still protects α-chymotrypsin from inactivation[18] by reaction with iodomethane but the protection is not 100% as in the present investigation where no anions or cations were added before lyophilization.

In summary, our results provide direct chemical evidence that the imprinting phenomenon[12, 14-17] on lyophilized α-chymotrypsin affects the chemical properties of the active-site by inducing a catalytically favorable orientation and interaction of the active-site functional groups during lyophilization. The results we have obtained so far suggest that ligands, which interact to stabilize the aqueous protein structure upon lyophilization, either enhance or abolish the chemical reactivity of active-site groups. Whether or not these observations turn out to be true generally, nonaqueous chemical modification of proteins lyophilized in the presence of ligands should provide the opportunity to determine the chemical properties of enzyme active-sites and to carry out specific chemical modifications that could not be achieved in aqueous solution.

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