Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking

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Glutaraldehyde possesses unique characteristics that render it one of the most effective protein crosslinking reagents. It can be present in at least 13 different forms depending on solution conditions such as pH, concentration, temperature, etc. Substantial literature is found concerning the use of glutaraldehyde for protein immobilization, yet there is no agreement about the main reactive species that participates in the crosslinking process because monomeric and polymeric forms are in equilibrium. Glutaraldehyde may react with proteins by several means such as aldol condensation or Michael-type addition, and we show here 8 different reactions for various aqueous forms of this reagent. As a result of these discrepancies and the unique characteristics of each enzyme, crosslinking procedures using glutaraldehyde are largely developed through empirical observation. The choice of the enzyme-glutaraldehyde ratio, as well as their final concentration, is critical because insolubilization of the enzyme must result in minimal distortion of its structure in order to retain catalytic activity. The purpose of this paper is to give an overview of glutaraldehyde as a crosslinking reagent by describing its structure and chemical properties in aqueous solution in an attempt to explain its high reactivity toward proteins, particularly as applied to the production of insoluble enzymes.
solutions, at room temperature in the absence of a catalyst. The final product (structure VIII) was proposed to be a soluble tetramer or pentamer containing approximately one free aldehyde group per molecule formed through the intramolecular-intermolecular propagation polymerization with ring formation.

In 1968, Richards and Knowles (10) studied glutaraldehyde solutions by proton (H) nuclear magnetic resonance (H-NMR). The NMR data (i.e., types of protons and peak integrations) did not agree with those expected from dimer, cyclic dimer, trimer, or bicyclic trimer only, but rather was consistent with a mixture of polymeric forms of these oligomers as well as higher polymeric species. The authors concluded that commercial solutions were largely polymeric and contained significant amounts of α,β-unsaturated aldehydes (structure VI) that were able to form rings (structure VII) by loss of water molecules by aldol condensation. The structure VI represents the average structure of the unsaturated polymerized glutaraldehyde (α,β-unsaturated compound), and Hooper (31) reported that the pendent aldehyde groups of structure VI would be scarcely hydrated because the carbonyl form is stabilized by conjugation.

Hardy et al. (21), in 1969, used ultraviolet (UV) spectrophotometry in combination with H-NMR to investigate glutaraldehyde solutions. They also found α,β-unsaturated aldehydes (structure VI), but only as a very minor component of the organic content because of the relatively weak absorbance observed at 235 nm. Furthermore, after purification of glutaraldehyde by liquid extraction with ethyl ether, these authors obtained 50% pure glutaraldehyde (structure I) with the expected H-NMR spectrum. Additional H-NMR investigations showed that purified glutaraldehyde undergoes very rapid hydration upon dissolution in water, which agreed with the results of Aso and Aito (20,30). Thus, Hardy et al. (21) postulated that glutaraldehyde monomer (structure I) existed as a mixture of hydrated forms (structures II, III, and IV) in aqueous solution, all of them being in equilibria.

In their work a few years later, Korn et al. (22) did not find significant amounts of α,β-unsaturated aldehydes (structure VI) by UV-spectrophotometric analysis, which was in agreement with the findings of Hardy et al. (21). They proposed, after H-NMR analysis, that aqueous solutions of glutaraldehyde consisted of free glutaraldehyde (structure I), the cyclic hemiacetal of its hydrate (structure IV), and oligomers of this (structure V), all in equilibria and in different proportions as a function of the temperature. In 1974, Whipple and Ruta (32) studied aqueous glutaraldehyde using $^{13}$C-NMR and concluded that aqueous glutaraldehyde consisted primarily of the cyclic hemiacetal (structure IV), but they added that this form was equally distributed between two geometrical isomeric forms (cis and trans).

Monsan et al. (23), in 1975, confirmed, for acidic glutaraldehyde solutions, the results obtained by Hardy et al. (21), Korn et al. (22), and Whipple and Ruta (32) using gel and thin-layer chromatography, mass spectrometry (MS), H-NMR, and infrared spectroscopy (IR). For glutaraldehyde solutions at neutral or slightly alkaline pH, they observed the production of molecular forms, which could precipitate. They also noted the formation of an abundant precipitate, named poly-glutaraldehyde, when sodium hydroxide was added to 25% aqueous glutaraldehyde solution to reach pH 11.0. MS and IR analysis allowed the identification of the solid as being the result of aldolic condensation of glutaraldehyde molecules (structures VI and/or VII).

In 1980, Margel and Rembaum (25) investigated the aldol condensation of glutaraldehyde in the pH range of 7.0 to 13.5. They obtained poly-glutaraldehyde and proposed the structure IX after spectroscopic and electrochemical analysis. They also reported that poly-glutaraldehyde may be soluble or insoluble in water and may have different concentrations of aldehyde, hydroxyl, and carboxylic functional groups depending on the polymerization conditions, such as pH and oxygen content, leading to the structures XII and XIII.

In 1991, Tashima et al. (27) obtained a new dimer when glutaraldehyde was treated by aqueous alkaline solution (pH 8.5). Their analyses by UV and IR suggested the existence of α,β-unsaturated
formyl and hydroxyl groups in this molecule, and gas chromatography (GC) MS analysis indicated the molecular formula $C_{10}H_{14}O_3$ (molecular weight: 182 g/mol). Moreover, after derivatization followed by two-dimensional NMR analysis, they proposed the structure X, in equilibrium with structure XI, for the glutaraldehyde dimer.

In 1992, Kawahara et al. (28) reported that most of the studies summarized above (10,21,23,32) neglected possible solvent effects on the glutaraldehyde structure. In fact, water is the medium in which commercial glutaraldehyde is supplied and in which the crosslinking reaction with proteins is carried out, and glutaraldehyde was found to react with this solvent in various ways. Thus, according to Kawahara et al. (28), there was a considerable problem in the studies carried out by Monsan et al. (23) because their analyses were conducted only in organic solvent (tetrahydrofuran for gel chromatography, chloroform/acetone for thin-layer chromatography, and deuterated chloroform or carbon tetrachloride for H-NMR). In fact, the equilibrium between monomeric and polymeric glutaraldehyde in anhydrous solvents could possibly shift to the latter to produce water. Other studies showed similar problems (10,21,32). For example, Richards and Knowles (10) and Hardy et al. (21) conducted their experiments in deuterated water, but the exchange of deuterium for hydrogen bound to the $\alpha$-carbon could give erroneous results when comparing the peak intensities by H-NMR (22). Moreover, the hydration equilibrium constants for monoaldehyde (formaldehyde) are reported to differ in water and deuterated water (33), and this probably occurs with glutaraldehyde. Whipple and Ruta (32) used $^{13}$C-NMR to analyze glutaraldehyde solutions, but direct comparison of the peak intensities is known to be not quantitative in $^{13}$C-NMR (34). Thus, these observations led Kawahara et al. (28) to investigate the molecular structure of glutaraldehyde in aqueous solution by UV absorption and light scattering. The 70% (w/v) glutaraldehyde solution used for their study was found to contain a large quantity of polymeric species with cyclic hemiacetal structure (V). Upon dilution, the polymerized glutaraldehyde slowly converted to monomers, thus inducing a great variation in the relative abundances of monomeric and polymeric species, according to the glutaraldehyde concentration. In fact, they found that in dilute solution and in the pH range of 3.0 to 8.0, glutaraldehyde was almost monomeric, predominantly in cyclic hemiacetal form (structure IV). In 1997, the same authors (29) found that glutaraldehyde structure was similar for aqueous solutions up to 10% (w/v) and concluded that the content of

Figure 1. Summary of the possible forms of glutaraldehyde in aqueous solution.
α,β-unsaturated structures (structure VI) was negligible regardless of the glutaraldehyde concentration.

In summary, several studies (10,20–29) have shown that commercially available glutaraldehyde represents multicomponent mixtures, but knowing which of these components is the most efficient for reactions with proteins is debatable. In fact, in aqueous solution, glutaraldehyde can exist in its simplest form, a monomeric dialdehyde, but also as a dimer, trimer, and polymer. Therefore, the effectiveness of glutaraldehyde immobilization and the controversies surrounding its chemical behavior could be rationalized with the multiplicity of structures, which depends on the solution conditions.

APPLICATION TO SUBSTANCES OF BIOLOGICAL INTEREST

Carbohydrates, Lipids, and Nucleic Acids

There is little information on the use of aldehydes to fix carbohydrates (35) or lipids (6). Most lipids do not react well with glutaraldehyde, with the exception of some phospholipids that contain primary amines (e.g., phosphatidylserine and phosphatidylethanolamine; Reference 36). In the case of nucleic acids, formaldehyde is by far the most effective agent for their fixation by reaction with amino groups of DNA nucleotides (37), but little information is available on the interaction between glutaraldehyde and DNA (38).

Proteins: General Case

Glutaraldehyde was used for the first time at the beginning of the 1960s for the fixation of tissues (1), and since this time many other applications have been developed. The high reactivity of glutaraldehyde toward proteins at around neutral pH is based on the presence of several reactive residues in proteins and molecular forms of glutaraldehyde in aqueous solution, leading to many different possible reaction mechanisms. Enzyme immobilization represents a good example to illustrate the use of glutaraldehyde as protein crosslinking reagent.

Glutaraldehyde can react with several functional groups of proteins, such as amine, thiol, phenol, and imidazole (39) because the most reactive amino acid side-chains are nucleophiles. Various data on aldehyde reactivity (at pH from 2.0 to 11.0) with the following amino acids have been reported in the literature: lysine (18); tyrosine, tryptophan, and phenylalanine (40); histidine, cysteine, proline, serine, glycine, glycylglycine, and arginine (41). These authors investigated the ability of different aldehydes to react with amino acids, and they ranked the reactive moieties of the amino acids in decreasing order of reactivity as follows: ε-amino, α-amino, guanidinyl, secondary amino, and hydroxyl groups. Avrameas and Ternynck (42) concluded that either glutaraldehyde did not react with the amine function of the guanidinyl group (arginine) or that in protein molecules the more reactive groups prevented the observation of arginine reactivity with glutaraldehyde. Okuda et al. (17) noted that glutaraldehyde reacted with thiol groups only in the presence of a primary amino group. Glutaraldehyde reacts reversibly with amino groups over a wide pH range (≥pH 3.0), except

![Figure 2. Schiff base (1) and Michael-type (2) reactions of glutaraldehyde with proteins.](image)
between pH 7.0 to 9.0 where only a little reversibility is observed (17).

The crosslinking of proteins, either to a carrier (solid support) or between protein molecules (carrier-free), generally implies the ε-amino group of lysyl residues (43). The unprotonated amino groups are very reactive as nucleophilic agents (44). It should be noted that lysyl ε-amino groups have pKa (acid dissociation constant) > 9.5, but it is presumed that the small percentage of amines present in their unprotonated form at lower pH is sufficient to react with glutaraldehyde, which then drives the acid-base equilibrium to deprotonation of these groups for further reaction. Most proteins contain many lysine residues, usually located on the protein surface (i.e., exposed to the aqueous medium) because of the polarity of the amine group. Furthermore, lysine residues are generally not involved in the catalytic site, which allows moderate crosslinking to preserve protein conformation (45) and thus biological activity (46). As stated previously, glutaraldehyde exists in multiple forms in aqueous solution, and all of these forms might be reactive toward lysine residues (ε-amino group) of proteins.

In spite of the substantial amount of literature concerning the use of glutaraldehyde, there is still no agreement about the main reactive species in glutaraldehyde solutions during the crosslinking process. Aldehydes are expected to form Schiff bases upon nucleophilic attack by the ε-amino groups of lysine residues in the protein (23). However, Schiff bases are unstable under acidic conditions and tend to break down to regenerate the aldehyde and amine. In contrast, the linkage formed by the reaction of glutaraldehyde with an amino group has shown exceptional stability at extreme pHs and temperatures, thus a simple Schiff base with both ends of monomeric glutaraldehyde has been ruled out as a mechanism for glutaraldehyde crosslinking with proteins. Several alternative mechanisms have been proposed.

Between 1968 and 1975, Richards and Knowles (10) and Monsan et al. (23) postulated pathways, both involving the reaction of the protein amino group with α,β-unsaturated aldehydes formed by aldol condensation of glutaraldehyde (Figure 2). Richards and Knowles (10) proposed that the reaction involved the conjugate addition of protein amino groups to ethylenic double bonds (Michael-type addition) of the α,β-unsaturated oligomers found in the commercial aqueous solutions of glutaraldehyde that are usually used (Figure 2, reaction 2). A few years later, Peters and Richards (47) showed work that supported this hypothesis because they found that, in the presence of an excess of amino groups, nucleophilic addition on the ethylenic double bond was possible. Monsan et al. (23) proposed a slightly different mechanism in which an addition reaction occurred on the aldehydic part of the α,β-unsaturated polymers (and poly-glutaraldehyde) to give a Schiff base (imine) stabilized by conjugation (Figure 2, reaction 1).

In the early 1970s, Boucher (48,49) proposed that monomeric glutaraldehyde was the active species involved in the crosslinking with proteins and that the facility of polymeric forms to revert to the active monomer depended upon pH (i.e., the type of glutaraldehyde polymers in solution). He also considered that polymers existing in the alkaline pH range cannot revert to the monomer because time and temperature tend to favor a more irreversible polymer, in contrast to polymers that exist at acidic and neutral pH. This was supported in 1990 when Ruijgrok et al. (50) showed that glutaraldehyde polymers in the neutral and acidic pH range could revert easily to the monomer under the influence of heating or ultrasonic radiation.

As early as 1976, Hardy et al. (51,52) and Lubig et al. (53) argued that the reaction of glutaraldehyde with proteins was not due to α,β-unsaturated aldehydes but may involve some dimer-
ization in the presence of the amino group, such as the formation of quaternary pyridinium compounds (Figure 3), rather than glutaraldehyde polymers reacting with amino groups. The mechanism involved could result from cyclization, dehydration, and internal redox reactions of a Schiff base. Hardy et al. (52) reported the isolation of a pyridinium-type compound following the reaction of glutaraldehyde with amines and suggested this structure as a stable crosslink. They showed that this compound had an UV absorption maximum at 265 nm, which was consistent with the original observation of Bowes and Cater (18).

In 1991, Tashima et al. (27) concluded that reaction of alkaline glutaraldehyde solutions (mixture of dimers X and XI) with proteins may involve a Michael addition to the double bonds to give (Xa) and (XIa), as shown in Figure 4 (adapted from Reference 19). If these reactions occur, no reduction is necessary to stabilize the adducts. If an excess of amine is added, the compound (XIb) may be formed.

In 1994, Walt and Agayn (19) proposed multiple reaction products for the different glutaraldehyde structures in solution depending on the pH conditions because each form of glutaraldehyde might participate differently in crosslinking reactions with proteins. Thus, under acidic or neutral conditions, glutaraldehyde exists as a mixture of monomers [i.e., free aldehyde form (I) or cyclic hemiacetal (IV)] or as a polymer [i.e., cyclic hemiacetal oligomer (V)]. Each of these structures would be expected to form Schiff bases upon nucleophilic attack by lysine residues in a protein, as shown in Figure 5. However, as previously mentioned, Schiff bases are unstable under acidic conditions and thus Schiff base formation (Figure 5, Equation 1) between a lysine amino group and free aldehyde (structure I) is not favored. It is more likely that monomeric cyclic hemiacetal (IV) and its multimeric form (V) react via reactions in Equations 2 and 3 of Figure 5, under acidic conditions. Under basic conditions (Figure 6), the reaction of α,β-unsaturated oligomeric aldehydes (VI) with amine can give two products robust to acid hydrolysis: a Schiff base (Figure 6, structure VIa), which was more stable because of the conjugation of the internal aldehyde group with the C-C double bonds and a Michael addition product (VIb). In the presence of excess amine, a mixed product (VIc) is seen, which is labile to acid hydrolysis because of the disruption of resonance stabilization. Because elimination of water in the formation of Schiff bases is reversible and because prolonged exposure to buffer solutions, particularly at elevated pH, might impair binding and lead to gradual release of the enzyme, reducing the double bonds of the Schiff bases by application of suitable reducing agents (19) such as sodium borohydride (NaBH₄) or sodium cyanoborohydride (NaCNBH₃) has been proposed. In both cases, the reduction of (VIa) produces a secondary amine that is tolerant to variations in pH and is stable even in acidic conditions. Sodium cyanoborohydride is preferred because it is a milder reagent (54). In fact, sodium borohydride not only reduces Schiff bases, but also aldehyde groups, leading to a lower yield of conjugate formation (55). Even if the use of a reducing agent has been recommended, reduction is not usually required (19).

In 1997, Kawahara et al. (29) speculated on whether proteins could catalyze the aldol condensation/polymerization of glutaraldehyde. They suggested that monomeric glutaraldehyde could be converted to polymeric forms by the action of amino groups and that this product played a major role in the crosslinking reaction of proteins. In fact, they proposed that the polymerization of glutaraldehyde via aldol condensation proceeded in parallel with the crosslinking reaction and that the formation of a Schiff base (imine) by one glutaraldehyde molecule with one amino group enhances its aldol condensation with other glutaraldehyde molecules. The final crosslinked structure would be a linear aldol-condensed oligomer of glutaraldehyde, with several Schiff base linkages branching off. They also observed that the dehydration step following aldol condensation occurs almost completely at the glutaraldehyde monomer units containing Schiff base imine, in contrast to the glutaraldehyde units containing no Schiff base, where little dehydration occurs. Therefore, the formed Schiff base linkage eventu-
ally constitutes a conjugate system with the adjacent ethylenic double bond. Once such conjugation is formed, the resonance interaction is reported to make Schiff base linkages stable to acid hydrolysis (23).

In conclusion, the chemical nature of the reaction of glutaraldehyde with proteins is not clearly understood, and the mechanisms of protein crosslinking reactions remain open to speculation. However, it seems that no single mechanism is responsible for glutaraldehyde reaction with proteins. In fact, because glutaraldehyde is present in different forms even for specific and controlled reaction conditions, several of the possible reaction mechanisms presented above could proceed simultaneously.

Enzymes

Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes or alternative technologies, and their applications are steadily increasing. Immobilization by covalent attachment to water-insoluble carriers via glutaraldehyde is one of the simplest and most gentle coupling methods in enzyme technology (43). The first reported application of the use of a bifunctional reagent was by Zahn in the 1950s (56), which was followed by studies on the chemistry of crosslinking with glutaraldehyde for the preparation of stable protein crystals for X-ray diffraction studies (45) or for the fixation of tissue samples for microscopic investigation (57). Later, glutaraldehyde was widely used as a mild crosslinking agent for the immobilization of enzymes because the reaction proceeds in aqueous buffer solution under conditions close to physiological pH, ionic strength, and temperature. Essentially, two methods have been used: (i) the formation of a three-dimensional network as a result of intermolecular crosslinking and (ii) the binding to an insoluble carrier (e.g., nylon, fused silica, controlled pore glass, crosslinked proteins such as gelatin and bovine serum albumin (BSA), and polymers with pendant amino groups).

Immobilization can be achieved for many enzymes under a wide range of conditions, which should be chosen according to the specific results required. These conditions have often been determined by trial and error because insolubilization is critically dependent on a delicate balance of factors such as the nature of the enzyme (42, 58, 59), the concentration of both enzyme (60) and reagent (58), the pH (61) and ionic strength (62) of the solution, the temperature (63), and the reaction time (64).

The nature of the enzyme, particularly its lysine content, has an effect on its insolubilization by glutaraldehyde (42, 58, 59). Moreover, if only a small amount of enzyme is available or if extensive modification must be avoided, the addition of inert, lysine-rich protein (e.g., BSA) has been suggested by Broun (58).

As mentioned above, the concentrations of
enzyme and glutaraldehyde must be carefully considered to obtain water-insoluble enzyme derivatives via crosslinking (60); low concentrations of enzyme and glutaraldehyde tend to induce intramolecular crosslinking by enhancing the probability that glutaraldehyde functional groups will react with the same enzyme molecule (60). Thus, conditions should be chosen carefully to favor intermolecular crosslinking between enzyme molecules instead of unwanted intramolecular links, which could also be formed (58,65,66). Broun (58) reported that the amount of crosslinking agent used affects the degree or extent of crosslinking. He indicated that low concentrations of glutaraldehyde were not able to form sufficient crosslinkages to effect precipitation of the enzyme. At higher concentrations, the extent of crosslinking was high enough to form a tight structure by excluding water molecules to insolubilize the enzyme derivative. Chui and Wan (67) indicated that enzymatic activity was inversely proportional to the concentration of glutaraldehyde used because extensive crosslinking may result in a distortion of the enzyme structure (i.e., the active site conformation). With this distortion, the accessibility and accommodation of the substrate may be reduced, thus affecting the retention of biological activity. Furthermore, the relative concentration of enzyme to glutaraldehyde should also be considered (17). We found that crosslinking of the enzyme trypsin (EC 3.4.21.4) with glutaraldehyde could be achieved over a wide range of relative mole ratios in 50 mM sodium phosphate buffer at pH 6.8 but that the time required to commence precipitation ranged from 0.5 to 120 min for enzyme:glutaraldehyde ratios of 1:500 to 1:25, respectively (I. Migneault, unpublished data).

The reaction of glutaraldehyde with enzymes to give soluble and insoluble products has been extensively studied, and the reaction was shown to be pH-dependent (39). Jansen et al. (61) showed that the optimum pH for glutaraldehyde insolubilization varied from protein to protein. In fact, they observed that the pH values for the most rapid insolubilization of BSA, soybean trypsin inhibitor, lysozyme (EC 3.2.1.17), and papain (EC 3.4.22.2) were found to be nearly the same as the isoelectric points (pIs) of these proteins, whereas the formation of insoluble active chymotrypsin (EC 3.4.21.1) was most rapid at pH 6.2 (pI 8.6) and chymotrypsinogen A at pH 8.2 (pI 9.5). The existence of an optimum pH suggests the important role of the protein charge on the intermolecular crosslinking required for insolubilization. The charge on the protein may regulate crosslinking, which was maximal when the repulsive charges were minimal. Furthermore, Tomimatsu et al. (62) and Broun (58) concluded that the lower the ionic strength of the reaction medium, the more rapid the crosslinking of chymotrypsin. On the other hand, the choice of pH should also be taken into account regarding the reactivity of aqueous glutaraldehyde, most immobilizations being conducted in the neutral or slightly alkaline pH range.

The influence of temperature and reaction time on insolubilization of enzymes has been reported by Broun (58). In early reports on enzyme immobilization, the reactions were carried out at low temperature (4°C), which was preferred for labile molecules, but the immobilization process required long reaction times (6–18 h; Reference 59). Ottesen et al. (63) and Bullock (35) suggested that the reaction of glutaraldehyde with lysine residues was progressive with time, probably depending on the accessibility of the ε-amino groups. Currently, ambient temperature is used for glutaraldehyde immobilization of enzymes within 4 h or less (68,69).

The catalytic activity of water-insoluble enzyme derivatives prepared using multifunctional reagents such as glutaraldehyde can vary considerably (61,63,70) and has been shown to be dependent on the amount of crosslinking reagent used during insolubilization, as well as on other factors (71). Moreover, the kinetic behavior of immobilized enzymes is, in many respects, different from that of free enzyme in solution (72), these differences being related to the diverse microenvironments generated by the enzymatic hydrolysis of the substrate. Kinetic properties of soluble enzymes are expressed in terms of Michaelis-Menten parameters. In the case of immobilized enzymes, apparent kinetic properties are used because the overall kinetic behavior of the enzymatic preparation is the sum of isolated contributions of each individual enzyme. 

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molecule, which can be immobilized via different amino groups, leading to different exposures of the catalytic centers (73). Our work (74) on trypsin immobilization with glutaraldehyde either by covalent attachment to aminopropyl controlled pore glass (CPG) particles or by crosslinking of trypsin in solution showed an increase in the apparent Michaelis constant, $K_{M,\text{app}}$ (i.e., a decrease in enzyme-substrate affinity) relative to free trypsin, which was more pronounced for glutaraldehyde-crosslinked trypsin compared to CPG-trypsin. Thus, according to our results, the crosslinking procedure led to a more constrained enzyme. Furthermore, the shapes of the pH-activity curves depend on the nature of the products liberated as well as on the kinetic parameters. Changes in the specificity of certain immobilized enzymes have been reported. For example, glutamic transaminase (EC 2.6.1.1) crosslinked with glutaraldehyde lost its transaminase activity but was still able to form complexes with its antibody (75).

Stabilities (thermal, chemical, and mechanical) of water-insoluble enzyme derivatives have also been described (46,76). Most notably, thermal stability of immobilized enzymes has been shown to vary from greater down to a lesser extent relative to the native enzyme. The stability of an enzyme (protein) can typically be increased by crosslinking because intra- and intermolecular crosslinks lead to a more rigid molecule that can resist conformational changes (77). In fact, the covalent bonds created during the crosslinking reaction are stable, even in the presence of substrate or high ionic strength solutions (59). Moreover, pH and temperature can be varied over a wide range without dissolution or deterioration of the crosslinked crystals (78). The crosslinking confers mechanical advantages because fragile crystals become much more sturdy and robust, so that there is much less chance of damage during handling, while remaining permeable to dissolved solutes. Among enzymes, proteases such as trypsin are of great interest because of their numerous applications in many fields. However, most of the commonly used proteases are marginally stable in their soluble form, the prominent cause of their irreversible inactivation being autoproteolytic digestion. Therefore, stabilization by immobilization has been the subject of considerable research. For example, we digested denatured lysozyme using two immobilized trypsin preparations (enzyme either covalently attached to aminopropyl CPG particles or crosslinked with glutaraldehyde) and did not observe autoproteolysis (I. Migneault, C. Dartiguenave, J. Vinh, M.J. Bertrand, and K.C. Waldron, submitted data). Moreover, these immobilized...
trypsin preparations showed excellent digestion reproducibility based on liquid chromatographic and capillary electrophoretic peptide maps. Insoluble trypsin preparations were found to be considerably more stable than native trypsin in the alkaline pH range (79). Glassmeyer and Ogle (80) reported that an insoluble trypsin preparation could be used repeatedly without loss of activity and could be left standing in pH 8.0 buffer at room temperature for 40 h with only a 9% loss of activity. However, the activity of insoluble trypsin preparations was completely destroyed upon incubation at 100°C in pH 8.0 buffer for 15 min (80). Walsh et al. (81) described the enhanced chemical stability of crosslinked, monolayered trypsin in the presence of urea, and Habeeb (70) reported a higher stability of trypsin crosslinked with glutaraldehyde during continuous use for casein digestions.

The storage stability of several water-insoluble enzyme derivatives has been examined because an insoluble enzyme derivative should retain activity for considerable periods of time to be useful. For example, Jansen and Olson (64) reported that papain (EC 3.4.22.2) crosslinked with glutaraldehyde showed no detectable decrease in esterase activity after 5 months at 4°C. Glassmeyer and Ogle (80) stored insoluble preparations of trypsin in water at 4°C for several months and, in most cases, at most a 15% drop in activity was found. Silman and Katchalski (79) did not note a marked loss of activity after lyophilization and storage at room temperature. On the contrary, catalase (EC 1.1.1.6) crosslinked with glutaraldehyde showed a decrease of about 20% in its initial activity after 2 weeks at 4°C in aqueous solution, after which no further decrease in activity occurred after 5 months of storage (11). Thus, storage of immobilized enzyme preparations depends on the nature of the product and should be tested for each case.

The application of chemical crosslinking is multidisciplinary, ranging from basic protein biochemistry to applied biotechnology, engineering, and medicine. Since the 1960s, glutaraldehyde has been used to couple enzyme (and protein) to carriers such as cellulose materials (82), aminoalkylsilylated glass (83), polyacrylhydrazide (84), phenalanyl-lysine coated polystyrene beads (85), and polyethyleneimine treated magnetite (86). Immobilized enzymes are also used in biosensors (87), chromatographic packings and detectors (88), online solid-phase reactors (89), and in the field of medical diagnostics and therapy (90).

There is an ongoing interest in carrier-free immobilized enzymes, such as crosslinked enzyme crystals (CLECs; References 45 and 91), crosslinked dissolved enzymes (CLEs; References 64, 70, and 74) and crosslinked enzyme aggregates (CLEAs; Reference 92). CLECs results from the chemical stabilization of the crystalline lattice of enzyme molecules by glutaraldehyde, forming highly concentrated immobilized enzyme particles that can be lyophilized and stored for a long time at room temperature. CLEs are obtained by the crosslinking of dissolved enzymes, which leads to enzymes with enhanced thermostability. CLEAs were synthesized in a simple and effective way by physical aggregation of the enzyme penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11) using a precipitant (e.g., tert-butyl alcohol), followed by chemical crosslinking with glutaraldehyde.

A different utilization of glutaraldehyde’s crosslinking ability, first reported in the late 1970s, is related to the preparation of microspheres for a variety of immunological and therapeutic applications (24). Glutaraldehyde was used for the preparation of microspheres from proteins (e.g., gelatin) or alginate because of its excellent fixative properties. Gelatin microspheres have been widely evaluated as drug carriers (93). Unfortunately, gelatin dissolves rather rapidly in aqueous environments, making the use of this polymer difficult for the production of controlled and/or long-term delivery systems (94). Thus, the use of a crosslinking procedure that leads to the formation of nonsoluble networks within the microsphere wall is required to reduce dissolution and premature drug release (95). Sodium alginate microspheres have been crosslinked with glutaraldehyde in the presence of calcium chloride (67), and applications of alginate microspheres have been reported in medicine (96) as well as in agriculture (97).

CONCLUSIONS

The success of glutaraldehyde as a crosslinking agent is a result of its multicomponent nature, where several forms are present in equilibrium in the reagent solution at a given pH. However, the exact molecular composition of glutaraldehyde solutions, as well as which component is the most reactive, is debatable despite plenty of knowledge. As a result, the reaction mechanism of glutaraldehyde with protein amino groups is not clearly understood, as illustrated by the large number of mechanisms reported in the literature and summarized in this review. No single mechanism seems to be responsible for glutaraldehyde crosslinking with proteins. All reported forms of glutaraldehyde exhibit the ability to react and crosslink proteins, leading to the formation of a broad range of conjugates. A fairly rigid control of reaction conditions is needed to achieve efficient insolubilization of each different enzyme due to their structural variability. Nonetheless, the resulting enzyme derivatives generally show good stability and thus can be reused. Although partial enzyme inactivation due to chemical modification is often unavoidable, in most cases enough catalytic activity is retained. More work is needed to provide additional information on the exact structure of these crosslinked products.

COMPETING INTERESTS

STATEMENT

The authors declare no competing interests.

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