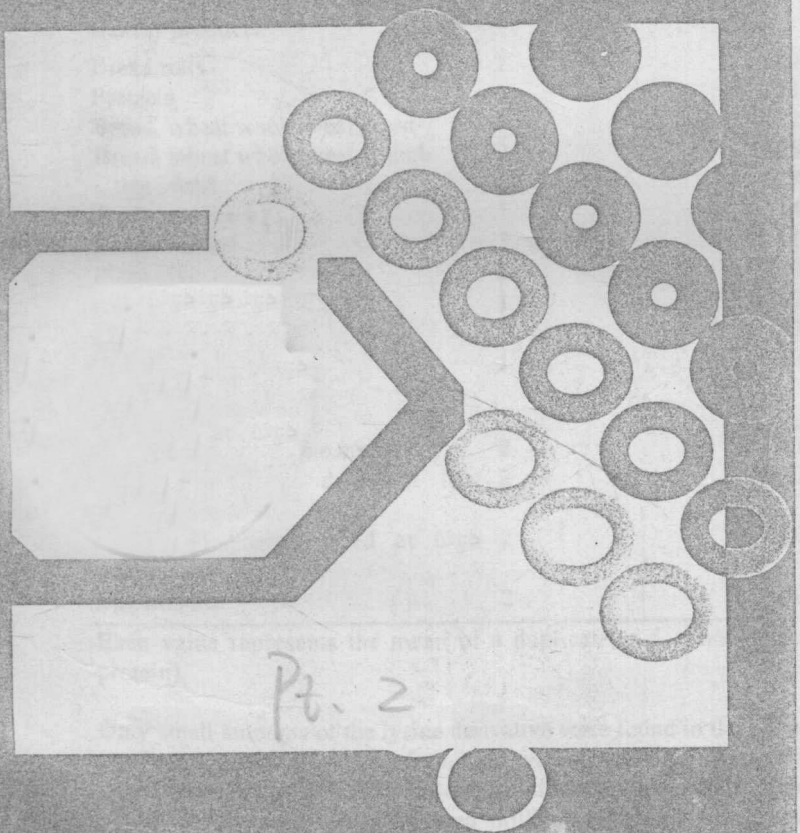
MAILLARD REACTIONS IN CHEMISTRY FOOD & HEALTH

PT. 2

MAILLAR REACTION IN CHEMISTR FOOD, AN HEALT



Edited by T.P. Labuza, G.A. Reinec
V.M. Monnier, J. O'Brien, and J.W. Ba

Table I. Pyrraline contents of commercially available foods.

Sample	Number of samples	Pyrraline (mg/kg protein)
Milk products		
Raw milk	2	n.d.
Pasteurized milk	2	n.d.
UHT-milk	2	n.d.
Sterilized milk	3	n.d. - 260
Evaporated milk	2	110; 130
Skim milk powder	4	n.d. - 150
	2	850; 1150
Whey powder	8	n.d.-90
	2	1450; 3150
Infant formula, powdered / liquid	5	n.d. - 160
Bakery products		
Bread rolls	2	20; 30
Pretzels	2	230; 240
Bread, wheat whole meal, crust	2	3250; 3680
Bread, wheat whole meal, crumb	2	25; 35
Crisp bread	2	280; 480
Rusk (zwieback)	1	1400
Pumpernickel	1	1130
Pizza	2	80; 110
Butter cookies	1	120
Wafers	1	570
Crackers	1	1320
Pasta products		
Italian spaghetti, home made	2	n.d.
Italian spaghetti, dried at low temperature	2	20; 40
Italian spaghetti, dried at high temperature	2	100; 125
Egg noodles	2	30; 50

Each value represents the mean of a duplicate; n.d = not detectable (below 2 mg/kg protein).

Only small amounts of the lysine derivative were found in the pasta products investigated, but here a greater pyrraline formation was observed in spaghetti samples dried at high temperature (up to 10 h at 85 °C) compared to low-heat products (up to 14 h at 55 °C).

In samples of severely heated milk powder and whey powder, four UV-active compounds with absorption maxima of 275 to 280 nm could be detected, eluting between phenylalanine and tryptophan in the amino acid chromatogram (Figure 2 a, b). Their formation correlated with heating time and temperature. By comparing retention time and UV-spectra with a standard sample, one of these compounds could be identified as maltosine, a pyridone derivative of lysine, which up to now has not been described in

protein hydrolysates. In contrast to pyridosine, another pyridone formed together with furosine from lysine Amadori products (Finot et al., 1981), protein-bound maltosine is a natural advanced Maillard reaction product of the 1-deoxy-glucosone pathway (Ledl et al., 1989). It is well known, that this route is favored at higher pH-values, whereas the degradation of the Amadori products via 3-deoxyglucosones and thus the formation of pyrraline proceeds mainly at pH-values between 5 and 7 (Ledl & Schleicher, 1990). This fact might explain the observation that rather small amounts of maltosine (about 100 mg/kg protein) compared to the corresponding pyrraline values (3150 mg/kg protein) were found only in a few browned samples.

Conclusion

With the proposed method it was possible to obtain information about the formation of protein bound pyrraline in foods. The lysine derivative may serve as a suitable indicator for the advanced stages of the Maillard reaction. As its content in some foods processed under the severe heating conditions of baking proved to be remarkably high, more information is necessary about the physiological properties of pyrraline as well as concerning the parameters influencing its formation. In contrast to pyrraline, protein bound maltosine represents only a minor compound of lysine derivatization during advanced browning. In order to clarify pathways of the Maillard reaction for proteins, isolation and structural identification of other amino acid derivatives is now in progress.

Acknowledgments
We thank Mrs. E. Hofmair for skillful technical assistance and Dr. H. Osiander, formerly at the Institut für Pharmazie und Lebensmittelchemie, Universität München, who kindly provided a pure standard sample of maltosine. This work was supported by a research grant from Deutsche Forschungsgemeinschaft.

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Catalytic Aspects of the Glycation Hot Spots of Proteins

Seetharama A. Acharya and Parimala Nacharaju

DIVISION OF HEMATOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE,
1300 MORRIS PARK AVENUE, BRONX, NEW YORK 10461, USA

Summary

A systematic investigation of the protein structural factors that could determine the chemical reactivity of the amino groups of proteins for nonenzymic glycation (glycation hot spots) has been carried out. The studies suggest that the microdomains of the glycation hot spots of proteins are Amadori rearrangement catalytic centers. The isomerization reaction catalysed by these microdomains is comparable to the reactions catalyzed by glucose isomerase and triose phosphate isomerase. The protein microdomains containing a constellation of positively charged functional groups thereby generating a proton rich microenvironment seem to act as the Amadori rearrangement catalytic centers. The α and/or the ε-amino groups located in such microdomains and accessible to aldoses to form the aldimine adducts are the glycation hot spots of proteins. It is suggested that the catalytic power of the glycation hot spots of proteins may have some role in the post glycation reactions leading to the generation of the advanced glycation end products and thus in the pathophysiology of diabetes.

Introduction

The nonenzymic glycation of proteins belongs to the class of *in vivo* post translational protein modification reactions that proceed spontaneously. The chemistry of the reaction involves the addition of glucose to the α or ε-amino groups of the protein through ketoamine linkages (Bunn et al., 1975). The glycation of proteins is initiated by the formation of a reversible Schiff base adduct of (aldimine) aldose with the amino group of protein. The aldimine adducts of simple aldehydes can be stabilized by reducing them using sodium cyanoborohydride or borohydride (Acharya et al., 1983). This results in the formation of an alkylated protein. Nature has developed an ingenious approach to stabilize the aldimine adducts of aldoses generated under

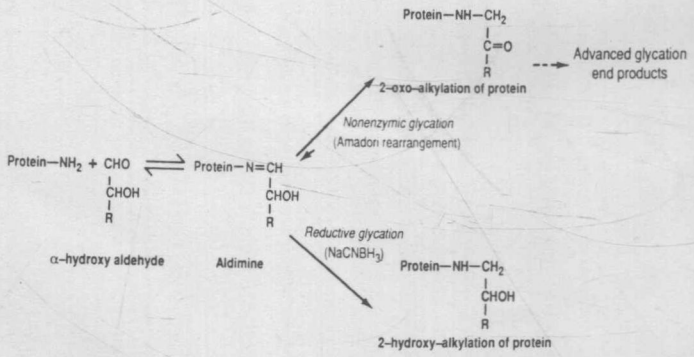

Figure 1. Schematic representation of reductive and nonreductive alkylation of proteins by aldose.

physiological conditions. This involves an intra molecular rearrangement of aldimine to a more stable ketoamine adduct (Amadori rearrangement). This is an alkylation reaction and the ketoamine adduct is a 2-oxo alkylated protein (Figure 1).

There is a high degree of site selectivity in the nonenzymic glycation of proteins; only a limited number of amino groups of a given protein undergo this modification reaction. These reactive amino groups are referred to as the glycation hot spots of proteins. The protein bound ketoamine adduct is the substrate for the post glycation reactions to generate the advanced glycation end products. Delineation of the unique structural aspects of the glycation hot spots of proteins has been the subject of considerable interest in recent years (Watkins et al., 1987; Iberg & Flukinger, 1986; Shilton & Walton, 1991; Nacharaju & Acharya, 1992).

Results and Discussion

The organic chemical aspects of the nonenzymic glycation reaction suggests that the reaction of α-hydroxyaldehydes with proteins should serve as a simple model for the nonenzymic glycation reaction. Consistent with this it has been now demonstrated that the aldimine adducts of other aldoses like glycolaldehyde, glyceraldehyde and erythrose also undergo the Amadori rearrangement. The open chain aldehydic form of glucose is the reactive species in the nonenzymic glycation reaction. However, the concentration of the reactive species of glucose under the physiological conditions is very low and accordingly the glycation reaction proceeds very slowly as compared with the lower homologues of aldoses, like glyceraldehyde. Aldodiose, aldotriose and aldotetrose are present as the reactive aldehydic form and react with proteins at a faster rate. Accordingly, we have used glyceraldehyde as the model aldose to delineate the molecular aspects of the initial phases of the nonenzymic glycation reaction. The site selectivity of the nonenzymic glycation of HbA and RNAse A with glyceraldehyde is comparable to that of glucose (Acharya & Manning, 1980), suggesting that the structure of the protein is the determinant of the site selectivity of the glycation reaction.

The nonenzymic addition of glucose to proteins will be facilitated at least by two structural aspects of the protein. First, a lowering of the pK_a of the amino group facilitates the glycation reaction as the unprotonated form of the amino group is the reactive species for the aldimine formation. The reaction could also be facilitated by endowing the microenvironment of an amino group a propensity to catalyze the isomerization of aldimine to ketoamine. A synergy between these two structural aspects is also possible. If the site selectivity of the glycation is a consequence of the pK_a of the amino group, the site selectivity of nonenzymic glycation and reductive glycation of a protein will be same as both of these reactions proceed through the same intermediate, the aldimine adduct (Figure 1). A comparative study of the reductive and nonenzymic glycation of HbA and RNAse A has established that the Amadori rearrangement step is the rate limiting step in the nonenzymic glycation of proteins.

An immediate question is to establish whether the potential of the glycation hot spots of proteins to catalyze the Amadori rearrangement (catalytic power) is the same. The site selectivity of the amino groups of HbA and RNAse A for reductive and nonenzymic glycation are different and this suggests that the Amadori rearrangement catalytic potential of glycation sites are distinct. An estimate of the catalytic power of the glycation sites has been obtained by comparing the extent of the chemical reaction at the glycation sites under the conditions of reductive glycation with that of nonenzymic glycation (Acharya

et al., 1991). In HbA the microenvironment of Lys-16(α) appears to be the most efficient in isomerizing the aldimine adduct to the corresponding ketoamine. The isomerization potential (catalytic power) of Lys-16(α) is about 650 times higher than that of Val-1(α), and 50 times higher than that of Val-1(β). Similar results have also been obtained for the glycation hot spots of RNAse A. The catalytic power of Lys 7, 37, and 41, are 750, 1300, and 1000 times higher than that at the α-amino group of Lys 1 of RNAse A.

The catalytic power concept invokes that a generality exists in the structural and/or conformational aspects of the glycation hot spots. The ultimate goal of all the studies on the mechanistic aspects of the site selectivity of the glycation reactions is to identify the unique aspects in the conformational design of the glycation prone amino groups of proteins and establish the commonality between them (nonenzymic glycation motif). The catalytic power at the glycation hot spots could be determined either by the amino acid sequence of the protein around the glycation site (nearest neighbor linear effect) or by the conformational aspects of the microenvironment of the glycation hot spots that bring unique functional groups of the protein to proximity (nearest neighbor three dimensional effect).

Nonenzymic glycation of performic acid oxidized RNAse A (Hirs, 1956) has given new insight as to which one of these two mechanisms best explains the site selectivity of the glycation reactions. The performic acid oxidized RNAse A lacks the tertiary interactions of the native protein, but retains the linear amino acid sequence of the polypeptide chain. Nonetheless, nearly 70% decrease was observed in the over all chemical reactivity of the amino groups of the protein for nonenzymic glycation as a result of performic acid oxidation. Interestingly, the influence of cleavage of the disulfide bonds of RNAse A on the chemical reactivity of all the glycation hot spots of the protein is not the same. Thus, the chemical reactivity of the glycation hot spots of RNAse A is not a consequence of the nearest neighbor linear effect.

On the other hand, nearest neighbor three dimensional effect appears to be the primary determinant of the site selectivity in the nonenzymic glycation of proteins. A comparison of the amino acid residues located in the microenvironment of the glycation hot spots of RNAse A and HbA suggested that the microdomains of proteins containing a constellation of positively charged functional groups, thereby a proton rich microenvironment, is the first approximate description of the Amadori rearrangement catalytic centers (Acharya et al., 1989). Amino groups (α or ε) located in such a region and accessible to form aldimine adducts with aldoses are the glycation hot spots of proteins.

A corollary to the nearest neighbor three dimensional effect concept is that the site selectivity depends heavily on the structural integrity of the native three dimensional structure of the proteins. A comparison of the nonenzymic glycation potential of Lys-16(α) of HbA in three different forms of the substrate has provided further support to the nearest neighbor three dimensional effect concept. When α-chain is used as the substrate for nonenzymic glycation, the influence of the quaternary structural aspects of the protein at Lys-16(α) will be absent. Similarly, the contribution of the tertiary and quaternary structural aspects of the protein to the microenvironment of Lys-16(α) will be absent when the segment $\alpha_{1\text{-}30}$ is used as the substrate. The microenvironment of Lys-16(α) exhibited a lower catalytic power in the α-chain, but had hardly any Amadori rearrangement activity in the segment $\alpha_{1\text{-}30}$. This establishes that the high catalytic power of the microenvironment of Lys-16(α) in the tetramer is not a direct consequence of the nearest neighbor linear effect (Nacharaju & Acharya, 1992).

Conceptually, the nearest neighbor three dimensional effect discussed above could be a direct consequence of a unique functional group (catalytic residue) located in the microenvironment of the glycation site or the catalytic power is an integrated property of a number of functional groups present in the microdomain. The unique functional group mechanism invokes an 'enzyme active site' concept to the catalytic centers. Mechanistically, the Amadori rearrangement reaction could be compared to the isomerization of the glyceraldehyde-3-phosphate to dihydroxy acetone phosphate that is catalyzed by triose phosphate isomerase or the isomerization of glucose to fructose catalyzed by glucose isomerase. The major difference is that the substrate is bound non covalently at the catalytic center in the enzyme systems where as in the protein glycation reactions the substrate (aldose) is covalently bound as the aldimine adduct at the catalytic centers. Therefore, it is tempting to speculate that the glycation hot spots of proteins represent 'degenerate isomerase-like active centers'. A corollary of the 'enzyme active site mimicry' concept is the implication of catalytic residues in the microdomains of the glycation hot spots. If this indeed is the case, the chemical modification or the site specific mutagenesis of such residues of the region will abolish the catalytic power of the microenvironment of glycation hot spots in much the same way as the chemical modification or mutation of the catalytic residues of the active sites does to the catalytic activity of enzymes.

A comparison of the general structural features of the microdomains of glycation hot spots of HbA and RNAse A revealed the presence of one or more His residues in the vicinity of the glycation hot spots. The His residues of the catalytic center could contribute to the catalytic power of the glycation hot spots by being only a contributor to the charge density of the domain. Alternatively the His residues could act as the catalytic residues of the Amadori rearrangement catalytic centers. Nonenzymic glycation of RNAse A carboxymethylated at its active site His residues has been undertaken to gain an insight into the possible role of the His residues in facilitating the glycation of Lys 7, 37, and 41. All of these three Lys residues are near the active site of RNAse A. His 12 and His 119 are the catalytic residues of the active site of RNAse A. Carboxymethylation of either one of these two His residues inactivates the enzyme. The carboxymethylation of one of these His residues of RNAse A could similarly influence the nonenzymic glycation catalytic power of this domain. The carboxymethylation of His-119 lowered the extent of nonenzymic glycation at Lys-7 and Lys-41, by nearly 70%, and that at Lys-37 by about 20%. The differential influence of the modification of His-119 of the domain on the chemical reactivities of three Lys residues of the region suggests the facilitation of the Amadori rearrangement by multiple structural factors (Nacharaju & Acharya, 1993).

The presence of residual amount of Amadori rearrangement catalytic power in the carboxymethylated RNAse A implies that His-119 by itself is not the sole contributor to the catalytic power of the glycation hot spots. The partial loss of the catalytic power of the glycation hot spots on carboxymethylation is comparable to the situations that generate partially active enzymes as a result of chemical modification. The interpretation put forward in such cases is that the modification introduces significant amount of conformational changes to the active site region of the enzyme, thereby decreasing the catalytic efficiency of the active site. If such conformational changes are invoked to explain the reduction in the chemical reactivity of glycation hot spots, it is also conceivable that these conformational changes could have influenced the pK_a of the ε-amino groups of this microdomain (Lys 7, 37, and 41). The observed loss in the chemical

reactivity of Lys residues may be an indirect consequence of changes in the propensity of the Lys residues to form aldimine adducts as a result of the chemical modification rather than being a reflection of the direct participation of the His residue in the catalytic process. A comparison of reductive glycation of carboxymethylated RNAse A with that of the unmodified RNAse A demonstrated that the propensity of the ε-amino groups of Lys 7, 37, and 41 to form the aldimine adducts is not significantly perturbed by the carboxymethylation of His-119. Thus, a direct role is implicated for His-119 in the catalytic power of the microenvironment.

In summary, these structural studies have established the basic concept, namely the role of the protein structure in the generation of protein bound ketoamine adducts. Further modification of these ketoamine adducts to generate the so called 'advanced glycation end products' (Figure 1) on one hand and the efficient removal of these advanced glycation end products by specific receptors has been suggested to determine the severity in the pathophysiology of diabetes. In view of the studies described here, it seems worthwhile to investigate the contribution of the structural elements of the microdomains of glycation hot spots on the formation of the advanced glycation end products.

Acknowledgements

This research is supported by a grant-in-aid from Juvenile Diabetes Foundation and the National Sickle Center award from NIH. We thank Professor R.L. Nagel for the facilities extended to us.

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Examination of Site Specificity of Glycation of Alcohol Dehydrogenase by Computer Modeling

D. J. Walton,[1] R. L. Campbell,[2] and B. H. Shilton[1]

[1]DEPARTMENT OF BIOCHEMISTRY, QUEEN'S UNIVERSITY, KINGSTON, ONTARIO, CANADA K7L 3N6; [2]INSTITUTE FOR BIOLOGICAL SCIENCES, NATIONAL RESEARCH COUNCIL, OTTAWA, ONTARIO, CANADA K1A 0R6

Summary

Molecular modeling was used to examine the site specificity of glycation of horse liver alcohol dehydrogenase in terms of structural features of the enzyme molecule. The results indicated that the catalyst for glycation of Lys-231 was likely to be the imidazole group of His-348, exerting its effect through the hydroxyl of Thr-347. For glycation of Lys-228, which requires phosphate, the base catalyst could be a phosphate ion, bound to the enzyme at a positive region of the coenzyme binding site. NAD^+ inhibited glycation of Lys-228 by binding to the enzyme and restricting access to glucose. Molecular modeling appears to be a useful tool for gaining and understanding the influence of protein groups, bound ions and bound coenzymes upon the site specificity of glycation.

Introduction

The site specificity of glycation of proteins has been ascribed to variations in the rates of Amadori rearrangement of the Schiff bases formed at different amino groups. The rate of rearrangement of a particular Schiff base is thought to be governed by the "isomerization potential" (Nacharaju and Acharya, 1992) of groups that are close to the glycation site.

Sequencing methods have been used to identify the sites of glycation of liver alcohol dehydrogenase (ADH) *in vivo* (Shilton and Walton, 1991) and *in vitro* (Walton and Shilton, 1991). The most significantly glycated amino groups are those of lysines 231 and 228, which are within the coenzyme binding domain. We now describe computer modeling studies that were conducted in an attempt to account for this site specificity.

Methods

Models of the Schiff bases formed by reaction of glucose with Lys-231 or Lys-228 of horse liver ADH were constructed with the Quanta program (Molecular Simulations Inc.). Torsional angles of (a) lysine side-chains, (b) side-chains of nearby amino acid residues, and (c) the sugar residue, were adjusted manually to optimize the relative positions of potential base catalysts and H-2 of the sugar. Atomic overlaps were avoided; energy minimization methods were not used.

Atomic coordinates were taken from Protein Data Bank file 6ADH (Eklund et al., 1981) for the holoenzyme containing NADH, and file 8ADH (Jones and Eklund, personal communication) for the apoenzyme.

Results and Discussion

Glycation of Lys-231

This reaction occurs *in vivo* (Shilton and Walton, 1991) and *in vitro* (Walton and Shilton, 1991), is relatively independent of the type of anion present in the solution, is unaffected by NAD^+, and is inhibited by guanidine hydrochloride (Walton and Shilton, 1991). It therefore seemed likely that rearrangement of the Schiff base formed at Lys-231 was promoted by nearby protein groups.

A preliminary examination of a model of holo-ADH showed that the amino group of Lys-231 is unique, as it is the only one of the thirty such groups that is within 8 A of a histidine (residue 348) imidazole group. It seemed possible that the latter promotes the Amadori arrangement of the carbohydrate of the Schiff base of Lys-231 by removing its C-2 proton. However, adjustments to the conformation of the peptide backbone would be needed to bring the imidazole and the attached sugar closer together. A more satisfactory rationalization of glycation of Lys-231 was obtained by the molecular modeling which is described next.

In a model of the holoenzyme containing a hexose residue attached to Lys-231, changes were made to the conformations of the sugar moiety, and the side-chains of Lys-231, His-348 and Thr-347. In the resulting structure (Figure 1, upper panel) the Thr-347 hydroxyl proton can form a hydrogen bond to one of the His-348 imidazole nitrogen atoms. The Thr-347 hydroxyl oxygen atom is in van der Waals contact with H-2 of the attached sugar. It was therefore possible to create an alignment of groups that would permit H-2 abstraction, without changing the conformation of the protein backbone. In this arrangement the basicity of the His-348 imidazole could be relayed to the sugar C-2 proton via the hydroxyl group of Thr-347. This would result in removal of the C-2 proton of the hexose to afford an enol, as shown in Figure 2. It therefore seems possible that the relatively high rate of glycation of Lys-231 is caused by catalysis of the Amadori rearrangement by side-chain groups of His-348 and Thr-347.

Modeling demonstrated that a bound coenzyme molecule, or a bound phosphate ion, would be too distant from Lys-231, Thr-347 or His-348 to affect the rate of glycation of Lys-231. Modeling also showed that glycation of Lys-231 would not be expected to be affected by a phosphate ion or a bound coenzyme molecule. The lack of glycation of Lys-231 occurring in the presence of guanidine hydrochloride was probably due to the distancing of this residue from Thr-347 and His-348 caused by denaturation.

Glycation of Lys-228

Glycation of the amino group of Lys-228, was interesting because if affected enzymatic activity by interfering with coenzyme binding (Walton and Shilton, 1993). Glycation at this site occurred only *in vitro*, and only if phosphate was included in the medium. The reaction was inhibited by NAD^+.

A phosphate ion can occupy the site of ADH that normally accommodates the coenzyme (Dahl and McKinley-McKee, 1980). Therefore the bound ion might be responsible for catalyzing glycation of Lys-228.

This hypothesis was tested by molecular modeling, as described next. The following procedure was adopted to produce a model (Figure 1; lower panel) of apo-ADH with an aldimine at Lys-228, containing bound phosphate. A phosphate ion was added to a model of the holoenzyme, in a position close to that normally occupied by the adenyl phosphate of NADH. A model of apo-ADH, with an aldimine at Lys-228, was added,

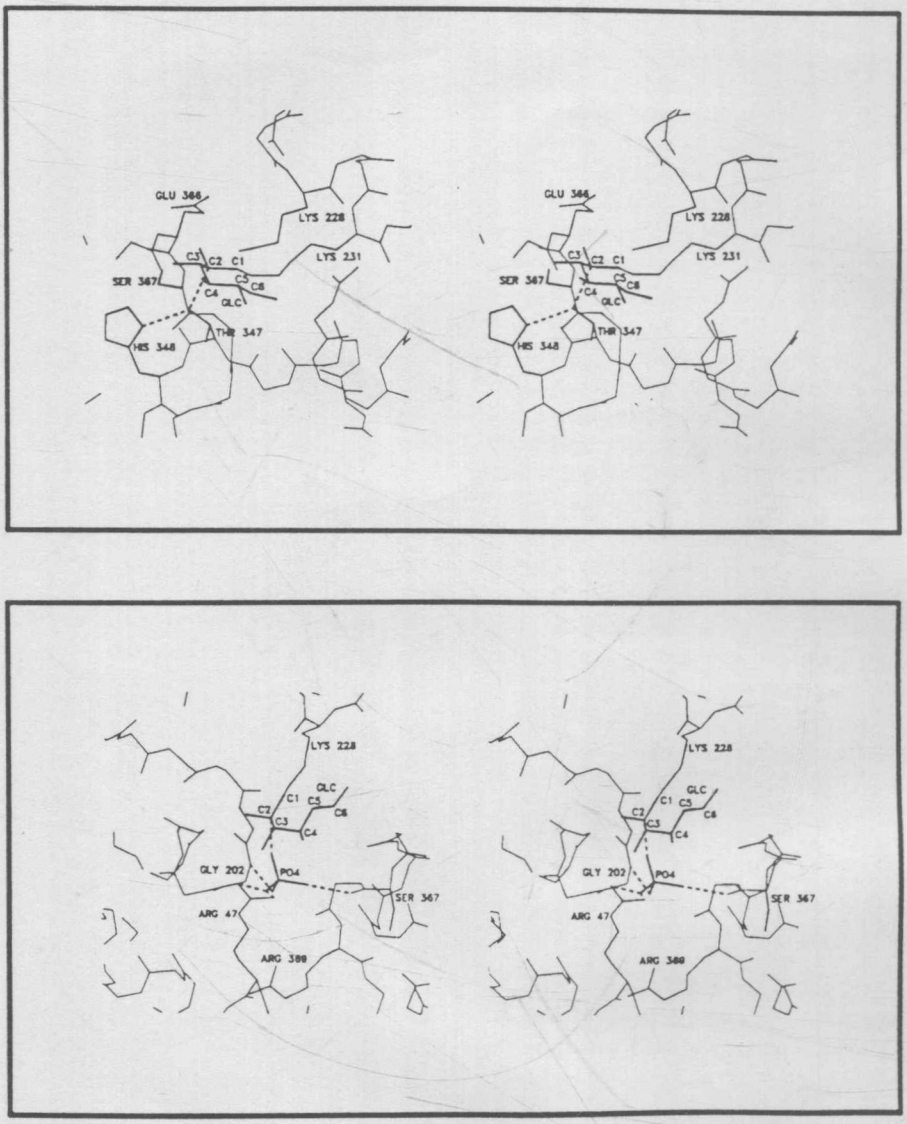

Figure 1. Views of portions of models of ADH derivatives. Upper panel: Holo-ADH, in which an aldimine has been formed by reaction of Lys-231 with glucose. The resulting model was modified as described in the text. Residues 228 and 231 are part of the coenzyme-binding domain. Other numbered residues are in the catalytic domain. Atoms that are likely to be involved in an Amadori rearrangement are joined by dotted lines. Lower panel: Apo-ADH, in which Lys-228 has formed an aldimine by reaction with glucose. A phosphate ion was added, and the model was adjusted as described in the text. Residues 47 and 367 are in the catalytic domain. Other numbered residues are in the coenzyme-binding domain.

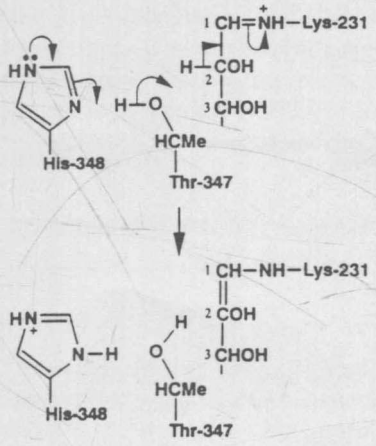

Figure 2. Charge-relay proposed to account for glycation of Lys-231.

and the coenzyme-binding domains of the two models were superimposed. The phosphate was then transferred to the apoenzyme model, where it was bound by two salt-links to Arg-47, and by hydrogen bonds to the amide hydrogen of Gly-202 and the carbonyl oxygen of Ser-367. After small adjustments to the torsional angles of the Lys-228 side-chain of the apoenzyme, it was easy to place one of the phosphate oxygen atoms at a position in which it would be connected to H-2 of the aldimine, via a hydrogen bond.

When ADH is in an aqueous solution, a bound phosphate ion may not be restricted to the position shown in Figure 1 (lower panel). However, the mode of binding shown in the model is feasible, as it involves several favorable contacts with the enzyme. The model demonstrates that phosphate could act as a base catalyst of an Amadori rearrangement, thus accounting for the phosphate-dependent glycation of Lys-228.

A model of holo-ADH showed that bound coenzyme would block access of the Lys-228 amino group to glucose, thus preventing glycation. The lack of glycation of Lys-228 *in vivo* (Shilton and Walton, 1991) can be explained in terms of the relatively low hepatic phosphate concentration, and/or protection by bound cofactors.

The pK_A values of the two catalytic groups invoked here, (7.2 for H_2PO_4 and 6.0 for histidine imidazolium) are close to the pH of the incubation medium (7.4). Hence they can act as acid-base catalysts of the interconversions of cyclic and acyclic tautomers that occur before, during and after glycation.

Conclusions

Previous attempts to account for the site-selectivity of glycation of a protein were based upon a consideration of the position of a reactive amino group in the primary (Arai et al., 1987; Baynes et al., 1989) or tertiary (Baynes et al., 1989; Nacharaju and Acharya, 1992; Shilton and Walton, 1991) structure. Since the critical step in glycation is the Amadori rearrangement, it is important to examine spatial relationships between potentially catalytic groups and a covalently-attached hexose, in the Schiff base form.

In the present study this was achieved by computer modeling. The method was used to demonstrate that interactions between a covalently-bound hexose, and side-chain groups of nearby amino acid residues (or a bound anion), could account for relatively rapid glycation at two locations in ADH. This new approach may prove to be useful in further studies of the site-specificity of glycation of proteins.

Acknowledgments

This work was supported by the Canadian Diabetes Association and the National Sciences and Engineering Research Council of Canada.

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Advanced Glycation Dependent Formation of Modified α-Crystallin and High Molecular Weight Aggregates

M. Cherian, A. Abraham, S. Swamy-Mruthinti, and E. C. Abraham

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, MEDICAL COLLEGE OF GEORGIA, AUGUSTA, GA 30912, USA

Summary

Modified α-crystallin (peak 3) fraction from 70-88 year old human lens protein was isolated by RP-HPLC and further analyzed by Sephacryl/guanidine HCl chromatography and SDS-PAGE and advanced glycated products were estimated by analyzing fluorescence spectra. Likewise, *in vitro* generated peak 3 from calf α-crystallin incubated with various sugars was isolated and studied. Highly fluorescent and cross-linked aggregates were present in both types of peak 3 preparations. Almost complete inhibition of peak 3 formation by aminoguanidine, an inhibitor of the advanced glycation process, suggested a direct involvement of advanced glycation in the formation of the peak 3 fraction.

Introduction

Human α-crystallin normally exists as a polymer having molecular weight of approximately 800,000 daltons. It undergoes yet unidentified modification becoming an aggregate of > 1 million daltons (Spector *et al.*, 1971). By applying reverse-phase (RP) HPLC we have shown earlier that the high molecular weight (HMW) α-crystallin fraction contains a predominantly modified α-crystallin peak (peak 3) with a longer retention time than either αB- or αA-crystallin (Swamy and Abraham, 1991). The same study also showed that the peak 3 component increases with lens age. In this report we show that the modified α-crystallin fraction is heterogeneous and contains cross-linked HMW aggregates enriched with advanced glycated products. *In vitro* glycation studies in the presence and absence of aminoguanidine (AG), an inhibitor of advanced glycation, suggested that the formation of peak 3 is advanced glycation dependent.

Materials and Methods

Calf lenses and 70-88 year old human lenses were decapsulated, homogenized in 50 mM phosphate buffer, pH 7.4 containing 50 mM NaCl (for calf lenses) or 50 mM Tris-HCl, pH 7.4 containing 0.2 M NaCl and 1 mM EDTA (for human lenses) and centrifuged for 1 hr at 10,000 g at 4°C and the supernatants were used for further studies. α-Crystallin fraction was purified by chromatography on a Sephacryl-S-300-HR column. About 30 mg of the protein was applied to a 80 x 1.6 cm column and developed isocratically with the respective homogenizing buffer. Calf α-crystallin was incubated with different glycating agents for up to 20 days in the presence or absence of 5 mM AG. Incubation mixture (0.5 mL) contained 2.5 mg α-crystallin in 150 mM phosphate buffered saline, pH 7.0 containing 0.5 mM EDTA 0.02% sodium azide and 1.5 mM phenylmethylsulfonyl fluoride. For the separation of αB- and αA-crystallin and the modified α or peak 3 fraction, RP-HPLC was carried out with a vydac C_4 column as decribed before (Swamy