Loss of Activity or Gain in Stability of Oxidases upon Their Immobilization in Hydrated Silica: Significance of the Electrostatic Interactions of Surface Arginine Residues at the Entrances of the Reaction Channels

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Abstract: The structural origin of the extreme variation of the effect of silica sol−gel immobilization on the stability of flavoprotein oxidases is examined. In glycolate oxidase and lactate oxidase, highly conserved positively charged arginine surface residues guide the substrate anions into channels containing the active sites. In glucose oxidase, both positively and negatively charged (arginine and aspartic acid) residues are found at the channel opening. We postulate that electrostatic interaction between the positively charged residues in lactate and glycolate oxidases and polysilicate anions disrupts the function or structure of these α-hydroxy acid oxidases upon their immobilization in hydrated silica. Such damaging interaction does not occur in glucose oxidase where the surface charges are internally balanced. The damaging electrostatic interactions of the α-hydroxy acid oxidases are avoided, and the enzymes are stabilized instead of being deactivated when complexed with polycations, prior to their immobilization in hydrated silica.

Introduction:

Means applied in stabilizing enzymes include their immobilization on solid hydrophilic surfaces[1−6] and cross-linking,[7−13] storage with nonvolatile polyols[14] or poly(ethylene glycol)s,[15] a means that is particularly effective for enzyme−polycation complexes,[16−18] and addition of salts, incorporation of salt bridges, and control of electrostatic interactions.[19−21] The concepts behind these stabilization strategies are (a) preservation of the structure of the oxidases through their cross-linking with a bifunctional reagent,[2,7,8,9,11] often through reacting surface amines of lysine and arginine residues with a reactive diester to produce diamides or with a dialdehyde to produce double Schiff bases; (b) preservation of their structure through encapsulation in a hydrophilic cavity, tailored to the size and shape of the enzyme, formed through a polymerization reaction;[2,3,10] (c) physical prevention of access of proteolytic enzymes to oxidases in the cores of the cross-linked aggregates and to those encapsulated in polymers; (d) reduction in the thermodynamic driving force for structural change, resulting from gain in entropy upon freeing of rotationally and translationally restricted, protein-bound, water molecules,[22] from global electrostatic interactions of charged surface functions,[23] or from gain in conformational entropy of chains.[24]

These concepts were successfully applied in the stabilization of glucose oxidase through its binding to a silica surface and encasement in hydrated silica gel, formed by the sol−gel

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GLYOX-So    ----MEITNVNEYEAIAKQKLPKMVYDYYASGAEDQWTLAENRNAFSRILFRPRILIDVT
HAOX-Rn     -----PLVCLADFKAHAQKQLSKTSWDFIEGEADDGITYSENIAAFKRIRLRPRYLRDMS
B2PRE-Pa    KPPLSQMINLHDFETIARQILPPPALAYYCSAADDEVTLRENHNAYHRIFFNPKILIDVK
MANH-Pp     --MSQNLFNVEDYRKLRQKRLPKMVYDYLEGGAEDEYGVKHNRDVFQQWRFKPKRLVDVS
LOX-Ms      GVAPTLPMSYADWEAHAQQALPPGVLSYVAGGSGDEHTQRANVEAFKHWGLMPRMLMAAT
LOX-Av      EIKYIDVVNTYDLEEEASKVVPHGGFNYIAGASGDEWTKRANDRAWKHKLLYPRLAQDVE
                       : .    : :.     :  . : *      *  .: :  : *:

GLYOX-So    NIDMTTTILGFKISMPIMIAPTAMQKMAHPE--GEYATARAASAAGTIMTLSSWATSSVE
HAOX-Rn     KVDTRTTIQGQEISAPICISPTAFHSIAWPD--GEKSTARAAQEANICYVISSYASYSLE
B2PRE-Pa    DVDISTEFFGEKTSAPFYISATALAKLGHPE--GEVAIAKGAGREDVVQMISTLASCSFD
MANH-Pp     RRSLQAEVLGKRQSMPLLIGPTGLNGALWPK--GDLALARAATKAGIPFVLSTASNMSIE
LOX-Ms      ERDLSVELWGKTWAAPMFFAPIGVIALCAQDGHGDAASAQASARTGVPYITSTLAVSSLE
LOX-Av      APDTSTEILGHKIKAPFIMAPIAAHGLAHTT--KEAGTARAVSEFGTIMSISAYSGATFE
              .  . . *     *: :.. .           : . *:.    .     *: :  :.:

GLYOX-So    EVASTGP--GIRFFQLYVYKDRNVVAQLVRRAERAGFKAIALTVDTPRLGRREADIKN-R
HAOX-Rn     DIVAAAPE-GFRWFQLYMKSDWDFNKQMVQRAEALGFKALVITIDTPVLGNRRRDKRN-Q
B2PRE-Pa    EIADARIPGQQQWYQLYVNADRSITEKAVRHAEERGMKGLFITVDAPSLGRREKDMKM-K
MANH-Pp     DLARQCD--GDLWFQLYVIH-REIAQGMVLKALHTGYTTLVLTTDVAVNGYRERDLHN-R
LOX-Ms      DIRKHAGD-TPAYFQLYYPEDRDLAESFIRRAEEAGYDGLVITLDTWIFGWRPRDLTISN
LOX-Av      EISEGLNG-GPRWFQIYMAKDDQQNRDILDEAKSDGATAIILTADSTVSGNRDRDVKN-K
            ::          ::*:*     .     : .*   *   : :* *    * ^  *    .

GLYOX-So    FVLPPFLTLK-----------NFEGIDLGK--------MDKANDSGLSSYVAGQIDRSLS
HAOX-Rn     LNLEANILLK-----------DLRALKE-----------EKPTQS--VPVSFPK--ASFC
B2PRE-Pa    FEADSDVQG------------DDEDIDR-----------SQGASRALSSFIDPS----LS
MANH-Pp     FKIPMSYSAKVVLDGCLHPRWSLDFVRHGMPQLANFVSSQTSSLEMQAALMSRQMDASFN
LOX-Ms      FPFLRGLCLTN-------YVTDPVFQKKFKAHSGVEAEGLRDNPRLAADFWHGLFGHSVT
LOX-Av      FVYPFGMPIV------------QRYLRG-----------TAEGMSLNNIYGASK--QKIS
            :                                                        .

GLYOX-So    WKDVAWLQTITSLPILVKGVITAEDARLAVQHGAAGIIVSNHGARQLDYVPATIMALEEV
HAOX-Rn     WNDLSLLQSITRLPIILKGILTKEDAELAMKHNVQGIVVSNHGGRQLDEVSASIDALREV
B2PRE-Pa    WKDIAFIKSITKMPIVIKGVQRKEDVLLAAEHGLQGVVLSNHGGRQLDYTRAPVEVLAEV
MANH-Pp     WEALRWLRDLWPHKLLVKGLLSAEDADRCIAEGADGVILSNHGGRQLDCAISPMEVLAQS
LOX-Ms      WEDIDWVRSITKMPVILKGIQHPDDARRAVDSGVDGIYCSNHGGRQANGGLPALDCLPEV
LOX-Av      PRDIEEIAGHSGLPVFVKGIQHPEDADMAIKRGASGIWVSNHGARQLYEAPGSFDTLPAI
             . :  :       :.:**:   :*.  .   .  *:  ****.^*      ..  *

GLYOX-So    VKAAQG-----RIPVFLDGGVRRGTDVFKALALGAAGVFIGRPVVFSLAAEGEAGVKKVL
HAOX-Rn     VAAVKG-----KIEVYMDGGVRTGTDVLKALALGARCIFLGRPILWGLACKGEDGVKEVL
B2PRE-Pa    MPILKERGLDQKIDIFVDGGVRRGTDVLKALCLGAKGVGLGRPFLYAMSSYGDKGVTKAI
MANH-Pp     VAKTGK-------PVLIDSGFRRGSDIVKALALGAEAVLLGRATLYGLAARGETGVDEVL
LOX-Ms      VKASGD-----T-PVLFDSGIRTGADVVKALAMGASAVGIGRPYAWGAALGGSKGIEHVA
LOX-Av      AERVNK-----RVPIVFDSGVRRGEHVAKALASGADVVALGRPVLFGLALGGWQGAYSVL
                       : .*.*.^^* .: ***. **  : :*^.  :. :  *  *   .

GLYOX-So    QMMRDEFELTMALSGCRSLKEISRSHIAADWDGPSSRAVARL------------------
HAOX-Rn     DILTAELHRCMTLSGCQSVAEIS----------PDLIQFSRL------------------
B2PRE-Pa    QLLKDEIEMNMRLLGVNKIEELTPELLDTRSIHNRAVPVAKDYLYEQNYQRMSGAEFRPG
MANH-Pp     TLLKADIDRTLAQIGCPDITSLSPDYLQNEG-VTNTAPVDHLIGKGTHA-----------
LOX-Ms      RSLLAEADLIMAVDGYRNLKELT----------IDALRPTR------------------
LOX-Av      DYFQKDLTRVMQLTGSQNVEDLKG---------LDLFDNPYGYEY---------------
              :  :    :   *  .: .:.
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Figure 1. Multiple sequence alignment of six members of the α-hydroxy acid oxidase family: glycolate oxidase from *Spinacia oleracea* (GLYOX-So), (*S*)-2-hydroxy acid oxidase from *Rattus norvegicus* (HAOX-Rn), cytochrome B2 precursor from *Pichia anomala* (B2PRE-Pa), L-(+)-mandelate dehydrogenase from *Pseudomonas putida*, and lactate oxidase from *Aerococcus viridans* (LOx-Av) and from *Mycobacterium smegmatis* (LOx-Ms). The alignment was carried out using the Clustal-W program (version 1.7). The first 180 residues of B2PRE-Pa, having no equivalent in six of the seven enzymes, were deleted, as were its last three residues. The first 17 residues of LOx-Ms and the first 12 residues of LOx-Av were also deleted for the same reason. Identical residues are marked with "*", highly conserved residues with ":", and moderately conserved residues with ".". The four highly conserved arginines residues are marked with "^". Sequences were taken from the Genbank Database. The numbering in the text follows that of the glycolate oxidase sequence.[38]

method,[25−31] the isoelectric point of which is near pH 5.[32] When freshly prepared and still highly hydrated, this gel is highly permeable to the reactants and to the products and immobilization does not lead to a loss of activity. The stabilization con-

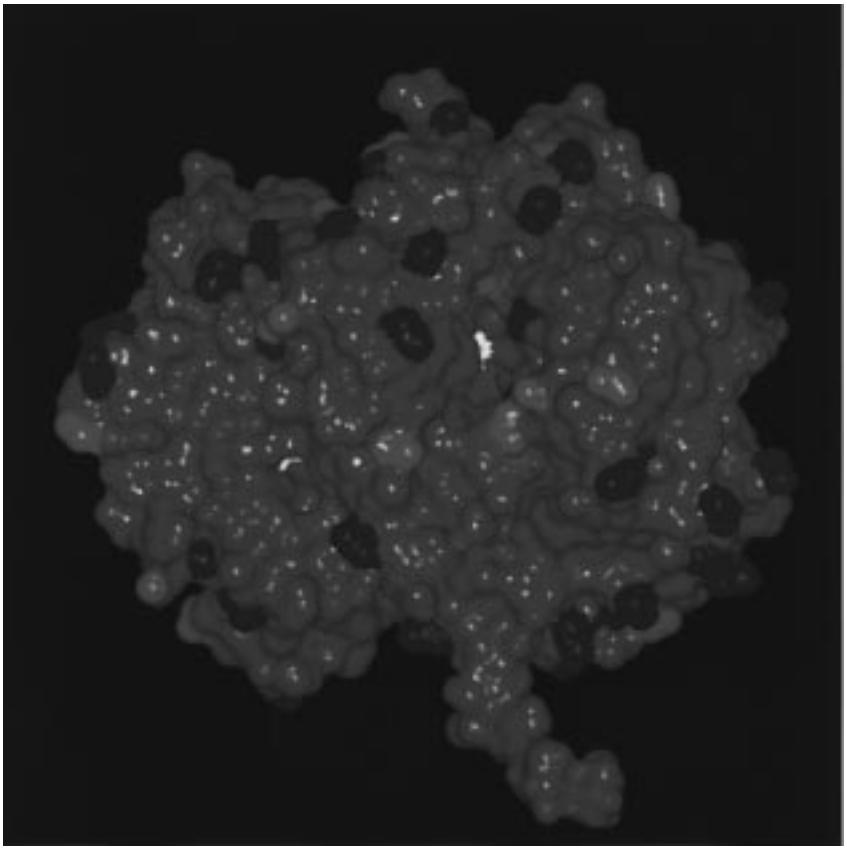

Figure 2. Connolly surface representation of the crystal structure of glucose oxidase. A ball radius of 1.4 Å was used. The active site FAD molecule, as defined by ref 35, is colored yellow. The C_z and NH side chain atoms of arginine residues and the N_z and NH side chain atoms of lysine residues are in red; the carboxylate functions of aspartate and glutamate residues are in blue. The charged residues of the entrance to the active site, listed clockwise from 12 o'clock, are R176, D416, D424, R512, and R335.

cepts that are valid for glucose oxidase are, however, not valid for either lactate oxidase or glycolate oxidase.[33] Of the three oxidases, only glucose oxidase retains its activity upon its sol−gel immobilization in hydrated silica and only glucose oxidase is stabilized upon such immobilization, its half-life at 63 °C increasing about 200-fold.[33] Lactate oxidase and glycolate oxidase lose most of their activity upon procedure-wise identical sol−gel immobilizations in hydrated silica, and the maintenance of their modest residual activity is not greatly improved upon immobilization. Formation of an adduct between glucose oxidase and poly(*N*-vinylimidazole) (PVI) or poly(ethyleneimine) (PEI) does not further improve the stability of glucose oxidase. However, adduct formation of lactate oxidase with PVI prior to sol−gel immobilization does prevent loss of activity and increases the enzyme's half-life at 63 °C 150-fold. In contrast, the stability of glycolate oxidase is not improved by adding PVI prior to immobilization, but when PEI is added, the half-life of glycolate oxidase at 60 °C also increases 100-fold.[33]

Here we relate the structures of the three oxidases with their stabilization. The model proposed is based on the known crystal structures of glycolate oxidase[34] and glucose oxidase[35] and upon the high degree of homology between glycolate oxidase and lactate oxidase,[36] the crystal structure of which has not been reported. We postulate that anionic silicate sites interact electrostatically with specific positively charged residues on the protein surface. When these positively charged surface residues are essential for either preserving the protein structure or for catalysis, their electrostatic interaction with silicate diminishes the activity.

Methods

A sequence alignment of α-hydroxy acid oxidizing apoenzymes having FMN cofactors, similar to but including more sequences from evolutionarily more diverse organisms than presented by Massey and his colleagues,[36] was performed using the program Clustal-W.[37] The sequences were taken from Genbank[38] and from Lindquist.[34] The crystal structures of glucose oxidase and glycolate oxidase were retrieved from the Brookhaven Protein Data Bank and viewed using Insight II (Biosym/molecular Simulations, Inc., San Diego, CA). Connolly surfaces were calculated using a ball of 1.4 Å radius.

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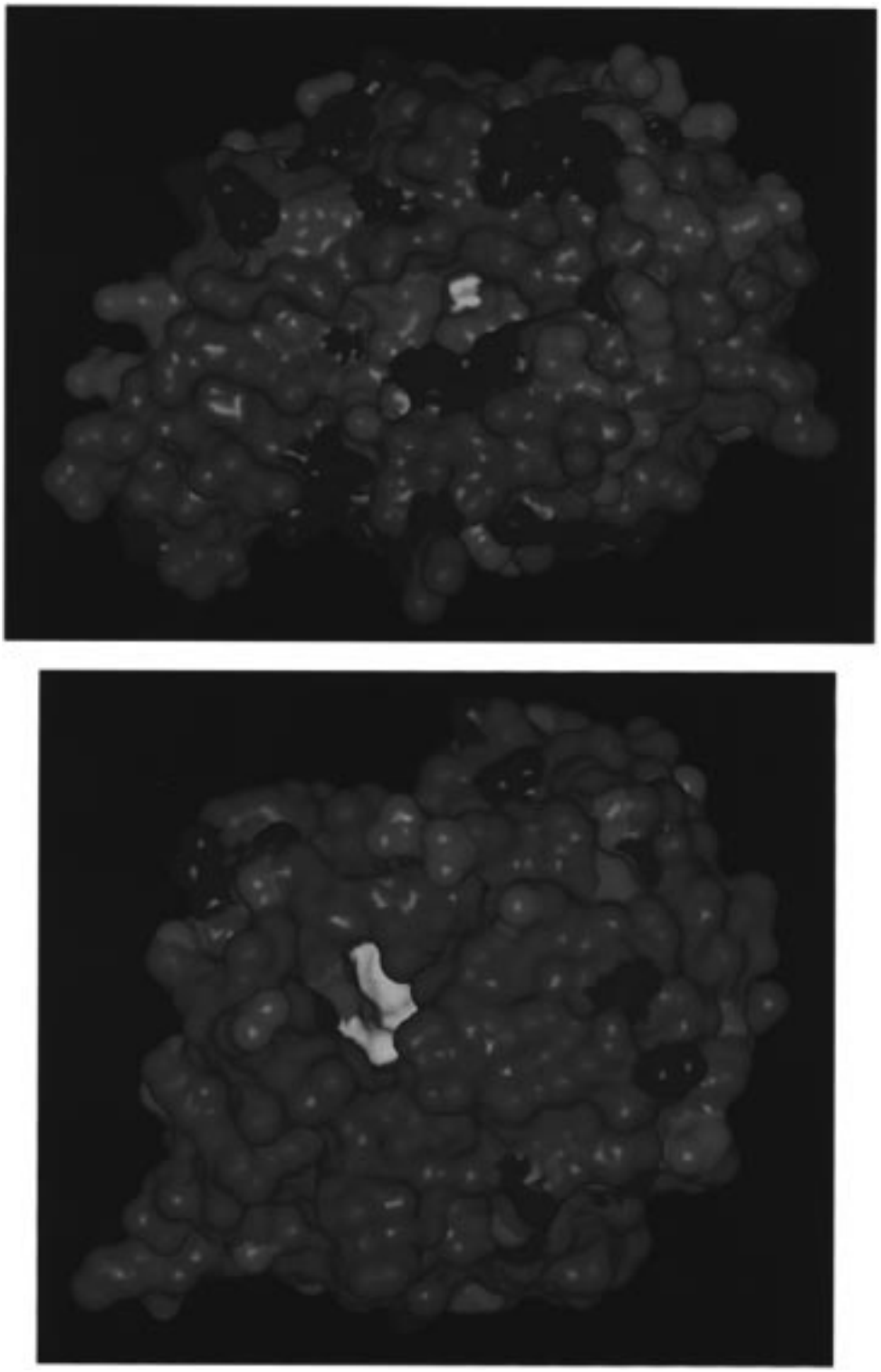

Figure 3. Connolly surface representation (ball radius = 1.4 Å) of the crystal structure of glycolate oxidase. Residues in the active site, as defined in ref 36, are colored yellow. The sequence alignment of α-hydroxyl acid oxidizing apoenzymes having FMN cofactors, similar to that presented by Massey and his colleagues,[36] was performed using the program Clustal-W.[37] C_z and NH side chain atoms of arginine residues and the N_z and NH side chain atoms of lysine residues are in red; the carboxylate functions of aspartate and glutamate residues are in blue. The reaction channel passes through the enzyme. (A, top) View of the larger entrance to the active site, surrounded by arginines 289, 290, and 309. (B, bottom) View of the small entrance surrounded by arginines 164 and 257 and aspartate 167.

Results and Discussion:

While glucose oxidase (pI = 3.8) lost little or none of its activity upon immobilization, lactate oxidase (pI = 4.6) and glycolate oxidase (pI = 9.6) lost at least 70% of their activities.[33] Glucose oxidase was shown to form an electrostatic complex with the copolymer of PVI with $[Os(bpy)_2Cl]^+$ complexed PVI.[39] Its stability in hydrated silica was not affected by

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precomplexing with PVI or with the more basic PEI. In contrast, lactate oxidase was stabilized by precomplexing with either PVI or PEI, and glycolate oxidase was stabilized by precomplexing with PEI, but not with PVI.[33]

Glycolate oxidase from *Spinacia oleracea* and lactate oxidase from *Aerococcus viridans* belong to a family of flavin mononucleotide (FMN) oxidases that catalyze the oxidation of L-α-hydroxy acids. Their amino acid sequences are 34% identical and 57% conserved. The sequence alignment of the two enzymes, along with that of other members of the L-α-hydroxy acid oxidase family, is shown in Figure 1. The crystal structure of glycolate oxidase has been previously determined to 2.2 Å resolution.[34] Because of the high degree of similarity between the sequences, the structures of glycolate and lactate oxidase can be assumed to be similar. Glucose oxidase is not a member of this protein family, belonging instead to a class of FAD-binding proteins. The structure of glucose oxidase from *Aspergillus niger* has been previously determined to 2.3 Å.[35] Connolly surface representations of the structures of glucose oxidase and glycolate oxidase are shown in Figures 2 and 3, respectively.

Both glycolate oxidase and lactate oxidase have conserved positively charged residues in and at the entrance to their FMN binding pockets. We postulate that the electrostatic interaction of these residues with polysilicate anions of hydrated silica leads to a decrease in activity upon sol−gel immobilization. When the protein surface is enveloped by a flexible polycation buffer, the silicate interacts with the buffer, not with the essential cationic residues. In this case the protein *is* stabilized by encasement in the silica gel. According to this postulate, PVI and PEI form adducts with lactate oxidase (pI 4.6), acting as buffers and preventing deactivation upon immobilization in silica. Glycolate oxidase (pI $\sim$ 9.6) is expected to form an adduct with the more basic PEI, but not with PVI.

The structure of the FMN binding site in α-hydroxy acid oxidizing enzymes has been discussed.[36] Residues R257, Y24, and Y129 (numbered according to the primary sequence of glycolate oxidase) are important for substrate binding, and H254 is necessary for catalysis. R257 is partially solvent-accessible (Figure 3). Maeda-Yorita, Massey, and colleagues[36] have shown that several of the active site residues are conserved in flavoprotein oxidases. The conserved residues include K230 and D 157, in addition to R257, Y24, Y129, and H254. As shown in Figure 1, the conservation holds broadly in all α-hydroxy acid oxidases that we examined, extending across enzymes from a wide variety of species, including higher plants and microorganisms. A high degree of conservation of residues in proteins is unlikely unless the conserved residues are necessary for a function common to members of the family or for preservation of their structure. Our analysis shows that residues R164, R289, and R309 are preserved in all of the L-α-hydroxy acid oxidases (Figure 1). The active site (shown in yellow in Figure 3) is located within a channel that passes through the protein. The larger opening is located on what we call the "front", while the smaller one is on the "back". Surrounding the front entrance to the channel are residues R289, R290, and R309 (Figure 3A), while the back entrance is surrounded by R164, D167, and R257 (Figure 3B). We postulate that some or all of the four conserved positively charged arginine residues on the enzyme surfaces are necessary for guiding the α-hydroxy acid anions into the active sites of the enzymes through an attractive electrical field on the protein surface.

Figure 2 shows a Connolly surface for glucose oxidase. The FAD molecule in the active site of the enzyme is shown in yellow. There are two arginine residues near the active channel of glucose oxidase. One comprises the group D424, R512, and R335; the second comprises D416 and R176. Thus, although positively charged residues (red colored) are present also in glucose oxidase, most of their charge is matched by negatively charged aspartic acid residues. With three positive surface charges balanced by negatively charged residues (blue color) in their immediate proximity and the third charge balanced by a slightly separated negatively charged residue, there is no prominent long-range electrical field. Indeed, because glucose is electrically neutral, such a field is not needed to guide glucose into the reaction channel.

When the positively charged surface residues in the L-α-hydroxy acid oxidizing enzymes interact with the negatively charged silicate, part of the protein may unfold or the geometry of the entrance to the active site may be disrupted. Either would result in a decrease in the activity of the enzyme upon immobilization in silica gel. Complexing the enzymes with a polycation prior to sol−gel immobilization prevents the damaging interaction. The flexible polycationic buffer on the enzyme shields the essential positively charged surface residues from the silicate anions, the negatively charged silica interacting with the shielding polycation[32] rather than with the protein.

The proposed stabilization mechanism may be put to test in two ways. According to the postulate, arginines at the reaction channel entrance are necessary for activity in lactate oxidase and in glycolate oxidase. Point mutants in which these arginines are replaced by either neutral or negatively charged residues should be inactive or less active than the wild-type enzymes. Furthermore, other members of the L-α-hydroxy acid oxidase family are expected to be stabilized in hydrated silica after precomplexing with polymers that are more basic than the enzymes.

Conclusions

Because electrostatic interactions between critical surface sites of oxidases and countercharged sites of rigid immobilizing matrixes can deactivate enzymes, immobilization in a rigid hydrophilic polyanionic matrix, even when its cavity is tailored for the size and shape of the enzyme, does not necessarily stabilize the enzyme. The detrimental electrostatic interactions can be overcome by complexing the enzyme with a polyelectrolyte that shields the critical charged sites. In the case of α-hydroxy acid oxidases, some or all of four positively charged surface arginine residues are essential for function or stability of the enzymes, and activity is lost when these interact with anionic sites of hydrated silica. If, however, these oxidases are precomplexed with a shielding, flexible polycation prior to immobilization, the enzymes are dramatically stabilized upon immobilization. In glucose oxidase, the most positive of the charges at the channel entrance are balanced by a negative charge of nearby acidic residues. For this reason, there is no detrimental electrostatic interaction with anionic sites of the immobilizing matrix, and the enzyme is stabilized upon immobilization in hydrated silica even when its positively charged residues are not shielded by a polycation.

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