Artificial Proteins

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Substrate Modulation of the Activity of an Artificial Nanoesterase Made of Peptide-Functionalized Gold Nanoparticles**

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Dedicated to Professor Richard W. Franck on the occasion of his 70th birthday

Enzymes have evolved to work at optimal efficiency within a precise interval of substrate concentration and, in many cases, the substrate itself controls the activity of the enzyme in order to modulate the metabolic cascade.[1] It would be desirable for an artificial enzyme to present not only the rate accelerations shown by natural enzymes but also these peculiar features. We have shown in the last six years[2–4] how gold nanoclusters passivated by a monolayer of functional thiols (Au-MPCs, MPC = monolayer-protected cluster)[5] may manifest exceptional catalytic properties connected with the characteristics of the functional groups of the monolayer and the multivalent nature of the system.[6] The behavior of Au-MPCs led us to coin the term "nanozymes" for these systems.[3] We report herein the outstanding case of peptide-functionalized Au-MPCs[7–13] that, like real enzymes, present unique catalytic pathways not present in analogous monomeric systems, including substrate modulation of activity during an esterolytic process.

The peptide sequence was designed according to the following principles. Ionizable residues are invariably involved in catalysis in nature. A combination of one His with two Arg and one Lys residue was expected to enable nucleophilic, general-acid, and/or general-base catalysis as well as stabilization of the negatively charged transition state that arises along the pathway of ester hydrolysis. Furthermore, charged residues are known to favor the stabilization of a peptide-capped nanoparticle.[11] In order to bring about substrate binding and proximity effects, hydrophobic residues were incorporated in positions buried in the core of the peptide assembly, with one Phe and one Tyr close to the key catalytic His residue. A Leu residue was also incorporated into the sequence, in the position closest to the nanoparticle, to further enhance the hydrophobic nature of the reactive site. The positions close to the spherical nanoparticle are severely constrained, and therefore an aliphatic side chain was favored over an aromatic one in the design. While there may be sequences that are capable of more efficient catalysis and a thorough search of chemical space might help to identify them, the number of combinations to screen becomes enormous even for short peptides and the process would require combinatorial strategies. Herein we have taken a rational approach in order to investigate whether a simple construct based on fundamental principles of organic reactivity would be enough to generate efficient catalysis.

Following these principles, we have prepared, by using standard solid-phase synthesis, five dodecapeptide-functionalized thiols[14] and used them for the passivation of Au nanoparticles by exploiting the site-exchange protocol and starting from nanoparticles covered with the water-soluble thiol *N*-(3,6,9-trioxadecyl)-8-sulfanyloctanamide (HS-C8-TEG).[15] In these five peptides the His catalytic site was moved along the sequence, with the aim of addressing the role of an increasingly hydrophobic environment in the catalytic process. Only the MPC functionalized with thiol **1** possessed the solubility and stability required for the kinetic investigation. This functional MPC, Au-PEP, contains HS-C8-TEG and peptide **1** in a 3:1 ratio, as determined by ^{1}H NMR spectroscopy; that is, there are approximately 62 peptides per nanoparticle on the basis of the average size (3.5 nm) of the gold core. Structurally this nanosystem resembles proteins both in size and in functionality.[16] The molecular weight of the organic monolayer is approximately 150 kDa.

Functional groups that may be involved in esterolytic activity of Au-PEP are the imidazole N atom of His (pK_a = 6.2), the phenol O atom of Tyr (pK_a = 9.7), the amine N atom of Lys (pK_a = 10.4), and the guanidinium N atoms of Arg (pK_a = 12).[17,18] Furthermore, the free carboxylate group of the C-terminal Arg residue may also be involved in a catalytic process. At physiological pH values (around 7) the most relevant functional groups are the imidazole unit of His which, because of its pK_a value, may act either as a general

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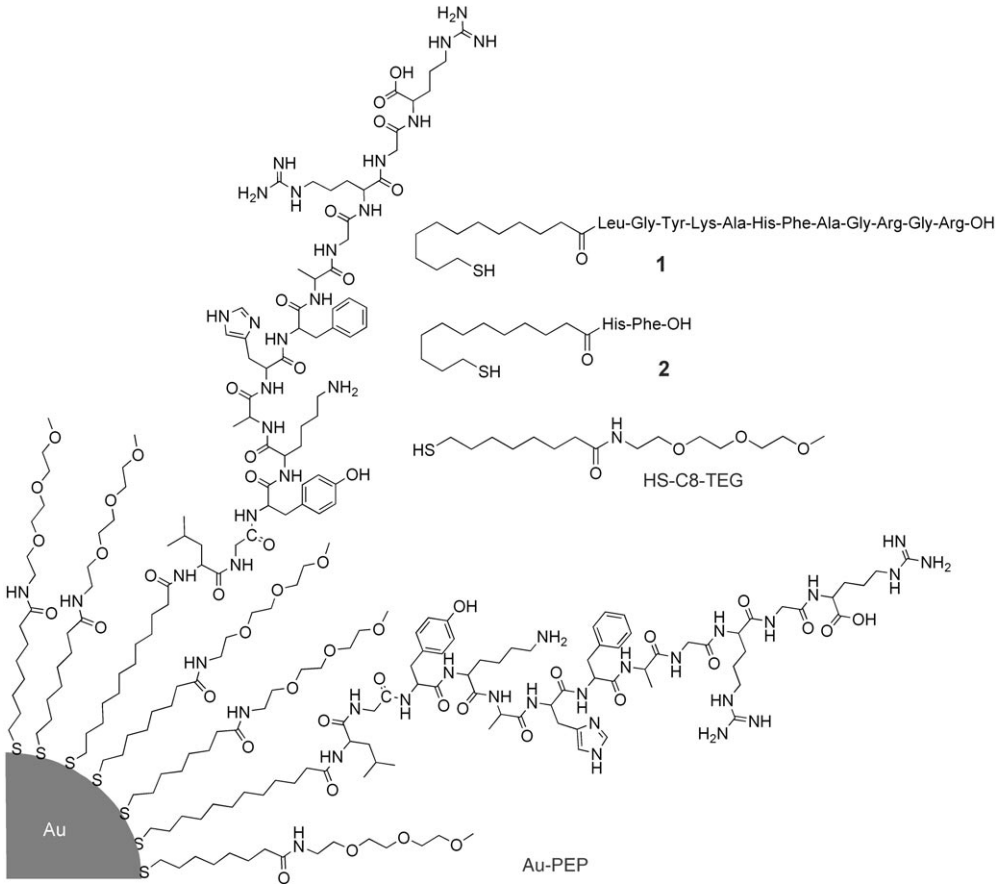
Supporting information for this article (including ^{1}H NMR spectra of the nanoparticles, high-resolution transmittance electron micrographs, and bimodal kinetic traces in the cleavage of Z-Leu-PNP) is available on the WWW under http://www.angewandte.org or from the author.



base–general acid or as a nucleophile and the C-terminal carboxylate which may act as a general-base catalyst. At variance with our previous dipeptide-functionalized nanoparticles containing thiol **2**[2] (functionalized with the dipeptide His-Phe-OH), the His residue in the present system is buried inside the monolayer and is probably in a less solvated region,[19] by analogy with what is typically the case for functional groups in the catalytic site of enzymes. From the structural point of view, the peptide sequence of **1** is not expected to fold into a particular conformation.

In order to test the activity of Au-PEP we have run kinetics studies with the activated substrate 2,4-dinitrophenylbutyrate (DNPB) and the *p*-nitrophenyl esters of benzyloxycarbonyl N-protected leucine and glycine (Z-Leu-PNP and Z-Gly-PNP, respectively; PNP = *p*-nitrophenol). DNPB is quite useful because the very low pK_a value of 2,4-dinitrophenol allows one to run kinetics studies down to pH values of 3–4 by following the absorbance of the 2,4-dinitrophenoxide ion at 400 nm (at lower wavelengths the molar absorbtivity of the nanoparticles is very high). Z-Leu-PNP and Z-Gly-PNP represent amino acid derivatives with high (the first one) and low (the second one) lipophilicities. They allow one to compare the role of their binding (and hence of their insertion into the putative catalytic site of the monolayer) in the catalytic process.

Figure 1 reports the activity, against the pH value, of the functional Au-PEP nanoparticles in the hydrolysis of DNPB, as $k_{2,app}$, the apparent second-order rate constant of the catalyzed process.[20] The profile is rather complex and evidences the contribution to catalysis of three nucleophilic species with apparent pK_a values of 4.2, 7.2, and 9.9. We attribute the first pK_a value to the terminal carboxylate anion, the second to the imidazole moiety of His, and the third to the amino group of Lys or the phenolic hydroxy group of Tyr. For comparison, we have also reported the profiles obtained with nanoparticles functionalized with peptide **2** (Au-**2**) and with S-acetylated peptide **1** (see Figure 1).

At low pH values the two nanoparticle-based catalysts behave in a similar way and catalysis depends on moieties with comparable pK_a values, most likely the terminal carboxylate anion. Up to approximately pH 5 Au-PEP is almost one order of magnitude more efficient than Au-**2**. This amounts to a 3000-fold rate acceleration over that exerted by the simple dipeptide AcHis-Phe-OH and a 500-fold acceleration over that of S-acetylated **1**. The most likely explanation for this behavior is that a carboxylate anion acts as a general base to activate a water molecule and a

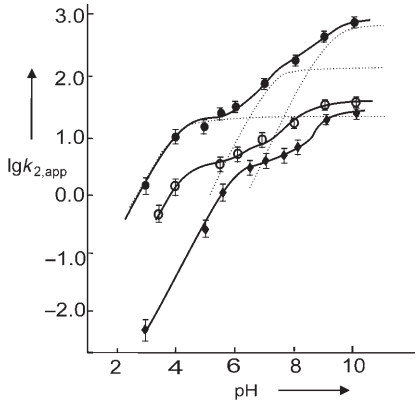


Figure 1. Logarithm of $k_{2,app}$ against the pH value for the hydrolysis of DNPB catalyzed by Au-PEP nanoparticles (●), Au-**2** nanoparticles (○), and S-acetylated peptide **1** (◆). The solid lines represent the best fits of functions describing the dissociation of residues involved in catalysis with pK_a values of 4.2, 7.2, and 9.9 for Au-PEP, 4.2 and 8.1 for Au-**2**, and 6.1 and 9.2 for S-acetylated **1**. The dotted lines represent the calculated contribution of each species to the solid curve for Au-PEP. Conditions: [catalyst] = 4.0×10^{-5} M, [buffer] = 10–20 mM, 25 °C.

protonated imidazole group acts as a general acid to form a strong hydrogen bond to the developing negative charge in the tetrahedral intermediate.[2] The larger catalytic efficiency of Au-PEP in comparison with Au-**2** arises from the stronger acidity of the protonated imidazole group (the pK_a value of the His of Au-PEP is 0.9 units lower than that of Au-**2**), as described by the Brønsted equation, $\lg k_{2,app} = A - \alpha pK_a$, where A is a constant and α is the Brønsted coefficient for general-acid catalysis. The observed difference in pK_a values between the His residues in the two systems is explained by differential interactions with other charged residues. While in Au-**2** the His residue is close to the carboxylate anion which increases the pK_a value of the imidazolium group, the three cationic Lys and Arg residues of Au-PEP give rise to a decrease in the His pK_a value due to electrostatic repulsion, in spite of the presence of the C-terminal carboxylate anion.

The His residue and the C-terminal carboxylate group are far apart in the sequence but the suggested cooperativity requires proximity in space. It is unlikely that the folding of peptide **1** in the monolayer covering the nanoparticle is such as to bring the carboxylate group of Arg into close proximity with the His residue. Accordingly, the most probable situation is that in which the carboxylate and imidazolium ions belong to two different, although spatially close, peptides. The suggestion that a carboxylate and an imidazolium ion are very close in the monolayer is also supported by the fact that the apparent pK_a value of the imidazolium ion is 1.1 units higher than that observed for S-acetylated **1**. In this peptide the protonated side chain of Lys completely shields the carboxylate effect on the pK_a value of the imidazolium ion which is, consequently, very close to the standard value reported for His. Thus, the experimental evidence indicates that the confinement of peptide **1** on the monolayer covering the surface of the gold nanoparticles significantly alters the properties of key functional groups involved in the catalytic process and triggers cooperative processes that are totally absent in the monomeric system.

As expected, the presence of the catalytic residue with an apparent pK_a value of 9.9 increases also the efficiency of Au-PEP in comparison to Au-**2** at the highest pH values studied. Thus, Au-PEP at pH 10 is almost 40-fold more active than Au-**2**. At this high pH regime either the amine group of Lys or the phenoxide moiety of Tyr may be responsible for catalysis. When the cleavage of DNPB was performed at pH 9.5 ESI-MS experiments indicated the formation of a butanoyl derivative of **1**. When treated with butylamine this derivative was completely deacylated, which suggests that Tyr and not Lys acted as a nucleophile.[21]

DNPB is an excellent substrate for studying the reactivity profile in the complete pH interval of 3–10 but it binds only weakly to the monolayer of Au-PEP. For this reason we have also studied the lipophilic substrate Z-Leu-PNP. However, in running kinetics studies with excess substrate to determine the Michaelis–Menten-like profile we observed bimodal kinetics: a first, fast burst of absorbance was followed by a slower process.[22] This behavior is typical of systems in which the catalyst is first transformed into an intermediate that is slowly cleaved to turn over the catalyst again. Since at pH 7 the relevant catalytic species is the imidazole group of the His residue of **1** anchored to the gold nanocluster, this intermediate is very likely to be the acylated imidazole group. Strikingly enough, the formation of this intermediate is not observed either with DNPB or Z-Gly-PNP, substrates that are less lipophilic (Z-Gly-PNP) or more reactive (DNPB) than Z-Leu-PNP. Furthermore, we had previously established that no intermediate was formed in the use of Au-**2** nanoparticles with Z-Leu-PNP as a substrate. Thus, the presence of both a lipophilic substrate (Z-Leu-PNP) and a catalytic site (His) buried inside the monolayer covering the nanoparticle apparently leads to a change in the rate-determining step of the hydrolytic process.

In order to get further insight into this intriguing behavior we have carefully analyzed the rate of formation of the intermediate and the rate of its hydrolysis working under pre-steady-state conditions (that is, with a catalyst concentration comparable to that of the substrate)[23] and the results obtained are reported in Figure 2. For Z-Leu-PNP the analysis reveals the following: a) the first step does not follow the typical Michaelis–Menten saturation kinetics but becomes faster as the substrate concentration increases, with a clear discontinuity at [substrate] $\approx 3 \times 10^{-5}$ M; b) the second step, that is, the breakdown of the intermediate, which should be independent of the substrate concentration, is also substrate-dependent and its rate monotonically decreases as the substrate concentration increases. Figure 2 also shows the behavior observed for Z-Gly-PNP, where there is no build-up of an intermediate: the initial rate of the cleavage process increases steadily as expected since, because of the poor lipophilicity of the substrate, we are far from saturation of the catalyst.

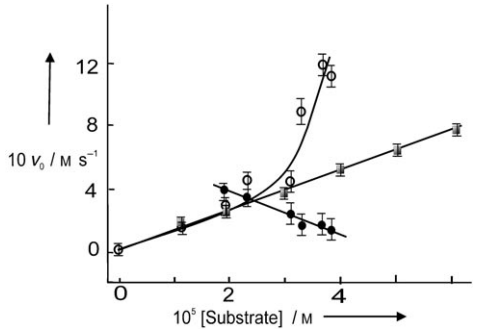


Figure 2. Dependence of the initial rates of intermediate formation (○) and of its hydrolysis (●) with Z-Leu-PNP and of hydrolysis (■) with Z-Gly-PNP upon substrate concentration. Conditions: [**1**] = 1.3×10^{-5} M (bound to Au-PEP), pH 7, 25 °C.

The data indicate that Au-PEP catalyzes with equal efficiency the cleavage of Z-Leu-PNP and Z-Gly-PNP up to a concentration of substrate of 3×10^{-5} M. Above this concentration the nucleophilic attack of the His imidazole group becomes much more efficient for Z-Leu-PNP than for Z-Gly-PNP. Paradoxically this does not result in an overall better performance of the catalyst because the cleavage of the intermediate becomes the rate-determining step and, under steady-state conditions, the hydrolysis is faster for Z-Gly-PNP than for Z-Leu-PNP. A possible explanation for this behavior

is that the binding of lipophilic Z-Leu-PNP alters the hydration of the catalytic site placed on the interior of the monolayer covering the gold core of Au-PEP.[19] A decrease in the water content may increase the nucleophilicity of the imidazole group by decreasing its solvation (thus increasing the rate of its acylation) and, on the other hand, may decrease the rate of hydrolysis (which depends on the concentration of water at the catalytic site). The binding of the substrate may also affect the structure of the catalytic site. Out of the three substrates we have investigated, selectively only one, Z-Leu-PNP, regulates the activity of the catalyst.

The present results indicate that several copies of a peptide bound to the surface of a gold nanocluster may lead to the formation of a functional nanoparticle with enzyme-like structure and properties. This is the first example of a protein-like system with considerable complexity yet self-assembled. Not only it is a good esterolytic catalyst but it is also capable of regulation of its activity. Thus, the anchoring of a functional peptide to the surface of a gold nanocluster results in a) the modulation of the properties of functional groups present on the side arms of the constituent amino acids, b) the induction of cooperativity between different groups at the catalytic site (an imidazolium and a carboxylate ion), and c) the creation of an environment different from the bulk solution and more similar to that found in the catalytic site of an enzyme (by depleting water molecules).[24] None of these properties is present in the monomeric peptide **1**, although all of the functional groups are obviously present in the oligomer. These are novel and striking features of these systems that compound with their multivalent nature[25,26] which has been shown already to lead to exceptionally high binding constants with selected substrates.[27]

Experimental Section

Synthesis of **1**: The peptide sequence of **1** was synthesized in the solid phase by using standard 9-fluorenylmethoxycarbonyl (Fmoc), *tert*-butoxycarbonyl (Boc), and *t*Bu protecting groups. Activation of the carboxylate was performed with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU). The hydrocarbon spacer terminating with the S-acetylated thiolic function was introduced in the last step just before removal from the resin with acidic treatment (95% trifluoroacetic acid (TFA)). The crude S-acetylated **1** was purified by HPLC (HICHROM 10C8 column), and the proper fractions were analyzed by MALDI-TOF mass spectrometry (*m*/*z* calcd for $[M^+]$: 1588.8781; found: 1588.996). Deacetylation was neatly performed by treatment with NH_2NH_2 in methanol.

Synthesis of Au-PEP: HS-C8-TEG-functionalized nanoparticles[15] (15 mg) were dissolved in N_2-flushed methanol (15 mL) in a jacketed reactor kept at 28 °C. Peptide **1** (9 mg) was added to the solution, and the reaction mixture was kept under an argon atmosphere with stirring for 72 h. The methanol was then partly evaporated, and the mixture was passed through a Sephadex LH-60 column with elution with methanol. Evaporation of the proper fractions afforded Au-PEP (22 mg). The exchange did not significantly alter the size of the original HS-C8-TEG nanoparticles ((3.4 ± 0.5) nm).

Kinetic experiments: Kinetics were studied under first-order conditions at (25 ± 0.2) °C and followed by monitoring the change of absorbance at 400 nm due to the release of 2,4-dintrophenolate or 4-nitrophenolate. Whenever necessary, absorbance was converted into concentration by using the molar extinction coefficients of the phenolates at a given pH value. Catalyst concentration when the nanoparticles are used as catalysts refers to the peptide component present on the monolayer. Typical catalyst concentrations were 1.3×10^{-5} M for reactions run with excess substrate and 4.0×10^{-5} M for reactions run with excess catalyst. Under pre-steady-state conditions the substrate concentration was varied between 1.0×10^{-5} and 4.0×10^{-5} M. The buffer concentration was 10–20 mM. The buffers used were *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS; pH 10.00), 2-(cyclohexylamino)ethanesulfonic acid (CHES; pH 9.00), 4-(2-hydroxyethyl)piperazine-4-(3-propanesulfonic acid) (EPPS; pH 8.00), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; pH 7.00 and 6.50), *β*-morpholinoethanesulfonic acid (MES; pH 6.00 and 5.5), acetic acid/sodium acetate (pH 4.00), and citric acid/sodium citrate (pH 3.00).

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