Reduction of Irreversible Protein Adsorption on Solid Surfaces by Protein Engineering for Increased Stability

Martin Karlsson‡, Johan Ekeroth§, Hans Elwing¶ and Uno Carlsson†.

Running title: Reduction of irreversible protein adsorption.

‡ IFM-Department of Chemistry, Linköping University, SE-581 83 Linköping, Sweden. § Current address: Eka Chemicals, Separation Products, SE-445 80 Bohus, Sweden. ¶ Department of Cell & Molecular Biology, Göteborg University, Box 462, SE-40530, Sweden. † To whom correspondence should be addressed. E-mail: ucn@ifm.liu.se Phone: +46 13 281714, Fax +46 13 281399

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The influence of protein stability on the adsorption and desorption behavior to surfaces with fundamentally different properties (negatively-, positively charged, hydrophilic and hydrophobic) was examined by surface plasmon resonance measurements. Three engineered variants of human carbonic anhydrase II were used that have unchanged surface properties but large differences in stability. The orientation and conformational state of the adsorbed protein could be elucidated by taking all of the following properties of the protein variants into account: stability, unfolding-, adsorption- and desorption behavior. Regardless of the nature of the surface there was correlation between (i) the protein stability and kinetics of adsorption with an increased amplitude of the first kinetic phase of adsorption with increasing stability; (ii) the protein stability and the extent of maximally adsorbed protein to the actual surface, with an increased amount of adsorbed protein with increasing stability; (iii) the protein stability and the amount of protein desorbed upon washing with buffer, with an increased elutability of the adsorbed protein with increased stability. All of the above correlations could be explained by the rate of denaturation and the conformational state of the adsorbed protein. In conclusion, protein engineering for increased stability can be used as a strategy to decrease irreversible adsorption on surfaces at a liquid-solid interface.

The adsorption of proteins at a liquid-solid surface interface has been of increasing interest because of its implications in medicine, biotechnology and the food industry (1,2). There are instances when protein adsorption is an unwanted effect, such as the irreversible protein adsorption that leads to biofouling of implantable biosensors and in biotechnological processes. There are also examples where protein adsorption is a desired feature, as long as this can be done in a controlled manner so that the structural and functional integrity of the protein is maintained. Examples of such areas are e.g. in production of combined, adsorbed vaccines (3) and in the development of chromatography material (4,5).

The areas where protein adsorption has a large impact can roughly be divided into two parts. First, where a specific surface is exposed to a large number of proteins, which includes bioimplantable materials and equipment for crude separation of proteins. In this case, the surface has to be engineered to avoid or govern the adsorption process (6,7,8). The second area is when a specific protein comes into contact with a large number of surfaces, e.g. in the downstream processing, polishing, storage and final use of proteins produced for biotechnological or medical applications. In this latter case it is unlikely that all of the surfaces (glass, plastic, stainless steel, rubber etc.) that a specific protein is exposed to during production and use, can be made resistant to protein adsorption; i.e. it could be more feasible to engineer the protein to make it less prone to...

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adsorb onto surfaces. Although this case has not been given as much attention, it is interesting to note that the downstream processing of, e.g., therapeutic antibodies accounts for an estimated 50-80% of the total manufacturing cost (9). Reduction of irreversible protein adsorption is likely to result in less biofouling and higher yields of protein and thus reduce the cost of downstream processing. Furthermore, it has been shown that protein adsorption phenomena can lead to a large loss of activity of therapeutically formulated protein solutions (10,11); i.e. there is both an economic and a safety incentive to reduce the adsorption of a specific protein that needs to be produced to meet biotechnological or medical needs.

It has been proposed that the stability of a protein is one of the determinants of adsorption behavior and can even be one of the driving forces for protein adsorption (12). The reason to this is that the conformation of folded proteins are quite restricted, i.e. the entropy is relatively low. However, if the protein upon adsorption tends to unfold to various degrees, this may lead to a conformational entropy gain, which can act as a driving force for adsorption even at hydrophilic surfaces under electrostatic repulsion. In terms of the degree of adsorption one can thus make a distinction between hard (stable) and soft (less stable) proteins. Therefore, one possible way to reduce protein adsorption could be to increase the thermal stability of a protein. In a recent study, this has also been shown to be the case, where sugar excipients were shown to decrease protein adsorption by stabilization of the protein native state in solution (13). Furthermore, conformational stability of proteins is an important determinant of the structure of the adsorbed protein (14,15), and earlier work employing point-mutated protein stability variants has described how the stability of a protein mainly influences with what rate the adsorbed protein undergoes conformational changes after adsorption (16). Thus, upon adsorption, a less stable protein will adopt various conformational states ranging from native, through molten globule, to a fully denatured state, each step giving rise to an increased number of interaction points between the protein and the surface and between the adsorbed proteins themselves (17). Hence, the irreversibility of protein adsorption will become more pronounced the more denatured the protein becomes when adsorbed to a surface. One possible way to reduce the irreversible adsorption of a specific protein could thus be to increase the thermodynamic stability of the protein and effectively make the protein more resistant to denaturation on the surface. In an earlier study (18), it was concluded that more stable variants of the same protein displayed a higher elutability upon elution with a detergent. However, the use of detergents is incompatible with the storage and use of, e.g., therapeutical proteins. Instead, in this paper we assess the influence that protein stability, as an intrinsic property of the protein, has on adsorption and desorption to four surfaces with fundamentally different properties (negatively charged, hydrophilic, hydrophobic, and positively charged). This will enable us to evaluate if (i) there is a correlation between stability and adsorption/desorption behavior and (ii) if this is independent of the surface’s properties. We have made use of three variants of human carbonic anhydrase II that differ in thermodynamic stabilities. Furthermore, the mutation sites for these three stability variants are made on the inside of the protein, i.e. other properties that can also influence the adsorption behavior such as size (19), surface potentials (20,21), hydrophobic patches (22) and secondary structure (23) are identical in the three variants. Thus, any differences in adsorption and desorption behavior originate in the stability of the protein variants and the different functionalities of the surfaces only.

The different surfaces were made up of self-assembled monolayers (SAM’s). In order to obtain well-defined surfaces, the monomers used to build up the SAM surfaces had the same "scaffold structure" onto which different functionalities were added. This minimizes any effects that might arise from e.g. variation in chain length, conformational flexibility (24) or monolayer order (25) etc. We have monitored the surface adsorption and desorption of the protein variants by surface plasmon resonance (SPR), an optical method that can be used to register the protein mass adsorbed.

MATERIALS AND METHODS
Surface Preparation. Synthesis of functionalized thiols—The synthesis of alcohol- (26) and sulphate- (27) terminated thiols have been described before. The methyl- and amino-terminated thiols were prepared according to the method for the alcohol-terminated thiol (26), substituting the aminoethanol for ethylamine hydrochloride and N-Boc-diaminoethane, respectively. The Boc-protection group on the amine was removed using trifluoroacetic acid, prior to thioester cleavage.

Gold Substrates—Gold substrates used for infrared reflection-absorption spectroscopy (IRAS), ellipsometry and contact angle goniometry were prepared as follows: Silicon (100) wafers were cut into appropriate sizes and washed in TL2, a mixture of water (milli-Q), 30% hydrogen peroxide (Merck, Darmstadt, Germany) and concentrated hydrochloric acid (Merck) (6:1:1) at 80 °C for 10 min. The substrates were then mounted in a UHV evaporation system (Balzers UMS 500 P) and primed with a 25 Å layer of titanium followed by 2000 Å of gold. The pressure was < 2×10⁻⁸ mbar during evaporation and the evaporation rate was 2 Å/s for titanium and 10 Å/s for gold.

Assembly—Prior to assembly, the gold substrates were cleaned in TL1, a mixture of water, 30% hydrogen peroxide and 25% ammonia (Merck) (5:1:1) at 80 °C for 10 min. The monolayers were prepared from ethanol (Kemetyl, Haninge, Sweden) solutions, having a thiol concentration of 1 mM, by placing the cleaned, gold surface in the solution for at least 15h. The substrates were removed from the solution, gently rinsed in ethanol followed by a five-minute ultrasonication in ethanol. Prior to use in experiments, the surfaces were dried under a stream of nitrogen.

Infrared Reflection-Absorption Spectroscopy (IRAS)—Infrared reflection-absorption spectra were recorded on a Bruker IFS66 Fourier transform spectrometer equipped with a grazing angle of incidence reflection accessory aligned at 85°. A liquid nitrogen cooled MCT detector was used. Interferograms were apodized with a three-term Blackmann-Harris function before Fourier transformation. The spectra were recorded by averaging 3000 interferograms (10 min) at 2-cm⁻¹ resolution.

Ellipsometry—Single-wavelength ellipsometry was performed on a Rudolph AutoEL ellipsometer. Light source was a He-Ne laser with wavelength 632.8 nm, at an angle of incidence of 70°. The fresh gold plates were measured prior to incubation with thiol to obtain reference values of clean gold. As a model, ambient/organic-film/gold, assuming an isotropic transparent organic layer having n = 1.5, was used. The film thickness was calculated by the AutoEL ellipsometer software as an average of three spots on each substrate.

Contact Angle Goniometry—Contact angles were measured with a Ramé-Hart NRL 100 goniometer with no control of ambient humidity. As contact liquids, freshly deionized water from a Milli-Q unit and hexadecane (Merck, Darmstadt, Germany) were used, respectively. Two separate measurements were performed on each plate.

Protein variants. For the assessment of the influence of protein stability to the adsorption and desorption behavior at surfaces, three stability variants of human carbonic anhydrase II were used. A pseudo-wild-type of HCA II (HCA IIpwt), with the mutation C206S, was used as a template for the S56N and A23C/L203C ox mutants. HCA IIpwt is indistinguishable from the HCA II wild type, regarding structure, activity and stability (28, 29). The production and properties of the S56N, HCA IIpwt and A23C/L203Cox variants have been described elsewhere (16,30,31,32).

Determination of protein melting temperatures (Tm)—Solutions of 0.85 µM protein in 0.1 M MOPS (3-(N-morpholino)propanesulfonic acid), pH 7.5 were prepared and kept on ice. 1.6 ml aliquots were transferred from the solution to a thermostated, 1-cm quartz cuvette. The time to reach unfolding equilibrium was established by monitoring the change in fluorescence intensity at 336 nm at each temperature and for each temperature a fresh sample was incubated in order to prevent any problems with aggregation during incubation. After the sample had equilibrated three fluorescence spectra (310-410 nm) of each sample were recorded.
Measurements were performed on a Fluoromax-2 spectrofluorometer (Jobin-Yvon Instruments). The excitation wavelength was 295 nm and the excitation and emission slits were 3 and 4 nm, respectively. The fraction of unfolded protein was calculated from the fluorescence data and plotted as a function of temperature. The transition curves were obtained from nonlinear least-squares analysis (33), using the program TableCurve 2D (Jandel Scientific).

Adsorption Experiments—All experiments were performed in 10 mM Na-borate buffer pH 8.5, prepared with ultra pure water (Milli-Q-plus system, Millipore, Bedford, MA). All the buffers were ultrasonically degassed under vacuum before use. In all experiments, the protein concentration was 50 µg/ml. SPR surfaces were cleaned prior to surface modifications in a UV/ozone chamber for 10-20 min. This was followed by immersion of the surfaces in a 1:1:6 mixture of H₂O₂ (30 %) (J.T. Baker), NH₃ (25%) (Merck) and Milli-Q water (Millipore) for 10 min at 65°C. The surfaces were transferred to a beaker of 95 % ethanol and cleaned from residual contaminants by ultrasonication for 1 min. The surfaces were then rinsed with ethanol before transfer to the respective thiol solutions. The surfaces were immersed in the respective thiol solutions overnight. SPR-measurements were performed on a Biacore 2000 system (Biacore AB, Uppsala, Sweden) providing a laminar flow through the measuring chamber. The flow rate was 25 µL/min and the temperature was kept constant at 22 °C. Gold substrates were gold covered glass plates supplied by Biacore AB treated as described under assembly before mounting in the Biacore-chip holder. The protein was introduced to the surface by passing a solution of the protein (50 µg/mL) in buffer over the surface for 13 min followed by automatic rinsing with buffer. The total measuring time was at least 43 min for duplicate or triplicate runs. Data collection was done using the Biacore 2000 software. For estimation of the surface-coupled mass to the flat surface, \( \Delta m_{\text{SPR}} \) (ng×cm\(^{-2}\)), we used the relationship \( \Delta m_{\text{SPR}} = C_{\text{SPR}} \Delta R_U \) where \( \Delta R_U \) is the difference in the dimensionless SPR response units and the constant \( C_{\text{SPR}} \) is 6.5×10\(^{-2}\) ng×cm\(^{-2}\) (34).

Treatment of Data for Evaluation of Adsorption Kinetics—All average adsorption isotherms were imported to the program TableCurve (Jandel Scientific) for kinetic evaluation. Three exponential terms were needed to make a good fit to the adsorption isotherms with, in some cases, an additional linear term.

Theoretically Adsorbed Mass—The experimentally determined masses adsorbed were compared to theoretical values in an attempt to gain additional information concerning the arrangement of the adsorbed protein. The values from the SPR measurements were compared with theoretical values calculated according to (i) a model assuming a close packed monolayer and (ii) a model based on random sequential adsorption (RSA).

In calculating the theoretical, close-packed monolayer for side-on and end-on adsorption the area occupied by each protein molecule is assumed to correspond to the circular footprint formed by rotating the long axis (55Å for side-on and 42Å for end-on) of the respective orientation. The RSA model assumes that the proteins approach the surface sequentially, are adsorbed irreversibly and do not diffuse laterally on the surface. The theoretical surface mass of such a model is about 54.7 % of a close-packed monolayer of spheres (35). Furthermore, the same calculations have been employed to calculate the theoretical monolayer coverage for the HCA II protein in its molten globule state by expanding each radius by 10 % (36).

RESULTS AND DISCUSSION

Protein variants—HCA II is a monomeric enzyme, with a molecular weight of 29.3 kDa, consisting of 259 amino acids. The protein is roughly ellipsoidal, with the dimensions 55 x 42 x 39 Å and is largely composed of \( \beta \)-sheets. The protein has a pI of 7.3 (37) and is expected to have a weak negative net charge at the pH chosen for the adsorption experiments (pH 8.5). The main features of the three protein variants used are summarized in Table 1, where it can be noted that all the mutations are situated inside the protein at locations that have no, or very low,
fractional surface accessibility, i.e. the surface properties of all the variants should be identical. This ensures that we have basically identical proteins that differ only in stability. The S56N variant has a lower thermodynamic stability, as compared to the HCA II pwt, whereas the oxidized variant of A23C/L203C (A23C/L203C_ox) has a higher thermodynamic stability. HCA II and all the variants that we have previously produced denature with a molten-globule intermediate that is stable at equilibrium. Such intermediates are characterized by a loss of tertiary structure, an increase in diameter (approx. 10%) (36) and exposure of hydrophobic patches (38). Interestingly, the stabilized variant A23C/L203C_ox has such an increased stability that its first transition from the native to the molten-globule state almost coincides with the second transition, the molten globule to the denatured state, giving an apparent two state transition (Fig. 1). This results in a markedly reduced tendency to form a molten globule state for A23C/L203C_ox. ANS-binding studies, during denaturation of A23C/L203C_ox in Gu-HCl, revealed that only about 10 % molten globule accumulated in solution, as compared with HCA II pwt (30). In an earlier study we showed that, upon adsorption to silica nanoparticles, HCA II pwt and destabilized variants thereof, formed a molten-globule-like state (14). This has later also been shown to be the case with other protein/surface systems (39). Furthermore, in the case of HCA II, it was shown that the more destabilized the protein, the faster this transition was from the native to the molten-globule state after adsorption (16). Although the A23C/L203C_ox variant denatures in accordance with an apparent two-state mechanism, all other properties resemble those of HCA II pwt. Thus, the near-UV CD spectra are almost identical, indicating a very similar tertiary structure and the enzymatic activity is about half that of HCA II pwt (30). The activity of S56N is very close to HCA II pwt and the mutation had no effect on the tertiary structure, as judged from CD analysis (16).

Surfaces—In Fig. 2, infrared reflection-absorption spectra (IRAS) are shown for the surface analogues assembled on gold. In addition to bands shown in Fig. 2 several bands were present at 2800-3100 cm⁻¹ (C-H, several differently functionalized hydrocarbons). The amide-I, -II and –III bands are visible for all surfaces at 1650, 1550 and 1260 cm⁻¹, respectively. For the terminal amine, the amide-I band coincides with bands from C-NH₃⁺. Characteristic sulfate bands are visible at 1250, 1080 and 1030 cm⁻¹. The ellipsometrically determined thickness is in good agreement with results from molecular modeling (Table 2). Values from contact angle measurements are also shown in Table 2. Values are within ranges expected for the present functionalities. Measurements from IRAS and ellipsometry showed that monolayers with the correct thickness for a 100% dense layer of the four analogues are formed on gold. The low absorption displayed for the amide-I stretch indicates that the transition dipole is oriented perpendicular to the surface normal, and thus having a molecular orientation of the carbon chain that is parallel to the surface normal (40). The difference in intensity of the amide-I stretch for the sulfate functionalized thiol is of low significance. The difference, however, indicates a slightly larger deviation from the surface normal orientation than for the corresponding methyl- and hydroxyl- functionalized molecules. Moreover, values from contact angle goniometry indicate that the derivatized group of the various assembled thiols (Fig. 2) are faced towards the environment. For convenience we will refer to the surfaces as negatively charged (-SO₄⁻), hydrophilic (-OH), hydrophobic, (-CH₃), or positively charged (-NH₃⁺).

Kinetics of Initial Adsorption—There appears to be no large differences in adsorption kinetics for the protein variants (Fig. 3A-D). This is what is to be expected, since regardless of protein variant, surface potentials etc. are identical between the three protein variants used. Some features of the adsorption kinetics are also common for all surfaces when analyzing the data. Firstly, as adsorption begins, the surface can be considered to be "infinite" and the kinetics during the initial adsorption is pseudo first order. The fitting of the data reveals that, regardless of surface, the amplitude of the initial phase is always lowest for the destabilized variant S56N, followed by HCA II pwt and the
more stable A23C/L203C_{ox} (Fig. 3 and Table 3). This can be explained by the faster rate that the less stable S56N variant changes its native state to a more denatured, expanded state after adsorption onto the respective surface. This has also been observed in an earlier study, where the rate of conformational changes was studied by CD upon adsorption of stability variants of HCA II to negatively charged silica nanoparticles (16). Because of the faster spreading of the less stable S56N, a larger surface is occupied within a shorter time, leading to lower amplitude of the initial phase. The more stable A23C/L203C_{ox} variant, on the other hand, has the largest amplitude of the three protein variants on all surfaces, implying that the structural integrity of the protein is maintained for a longer time after adsorption. Secondly, there is also a correlation between the stability of the protein variant and the rate constant of the initial adsorption. Although the differences are fairly small, the rate constants for the more stable A23C/L203C_{ox} variant are in all cases lower than for S56N and HCA II pwt during the initial adsorption, despite the fact that the amplitude for the initial phase of adsorption is the largest for A23C/L203C_{ox} and that the attractive forces between the respective surface and each protein variant are identical. Hence, the differences in the rate constants of the initial adsorption is supportive of the notion that an increase in entropy of the adsorbed state could be a driving force for adsorption and that a hard (stable) protein is less prone to adsorb to a solid surface at a solid surface/liquid interface (12). Furthermore, because the structure of A23C/L203C_{ox} is tethered by a disulfide bridge, even less entropy gain can be expected for this variant even if it looses its native conformation after adsorption.

**Adsorbed Mass and Orientation**—Under the experimental conditions used, all adsorption isotherms (Fig. 3A-D) display a continuous adsorption without any overshoot. The presence of an overshoot in adsorption isotherms has earlier been explained by reorientation of adsorbed molecules at the surface (41) or a displacement of adsorbed proteins by "competitive spreading" (42). The adsorbed mass differs substantially between the different surfaces and less within the protein stability variants. In most of the cases the more stable A23C/L203C_{ox} variant, with a melting point of 74°C (data not shown) and a reduced tendency to form a molten-globule state under denaturing conditions in solution (30), adsorbs to the largest extent. This is likely to be due to the fact that this variant undergoes less conformational changes during initial phases of adsorption, leaving more space for molecules, which adsorb subsequently. This also leads to the conclusion that a higher thermodynamic stability of a protein does not mean that such a protein will adsorb to any smaller extent as compared to a less stable protein. On the other hand, the least stable S56N also appears to adsorb to a large extent. However, as is evident from the kinetic analysis and the adsorption isotherms, this is most probably due to the formation of a second or additional layers of proteins forming on the surface of the already adsorbed protein molecules. This can be seen as a slow linear phase of continuous adsorption (Fig. 3A-C), which is not present in the adsorption isotherms of HCA II pwt and A23C/L203C_{ox} which reach a plateau value and stay constant after a few minutes of exposure to the protein solution on all surfaces except for the positively charged one (Fig. 3A-D). The slow linear phase displayed by S56N can be explained by the fact that S56N has a rather low stability in solution, with a melting point of 46°C and an unfolding transition that starts at approx. 10°C below this value (data not shown). Thus, some of the protein molecules in solution are likely to form aggregates with the adsorbed S56N, which in the adsorbed state rapidly forms molten-globule-like structures (16). Indeed, such molten-globule states of HCA II have been shown to be very prone to aggregate (43). This starts the formation of an additional layer of proteins, which makes up the final, slow linear phase. If it were not for the additional protein layers formed, it is reasonable to believe that the least stable S56N variant would adsorb to the smallest extent during the time span observed. HCA II pwt with the intermediate stability of the three variants, also forms a molten-globule state, although not as fast as the S56N variant (16). The thermostability of HCA II pwt is considerably higher than for S56N, with a melting point of 74°C.
approx. 59°C (data not shown), and as can be seen in Table 3, the kinetic adsorption amplitude of the HCA II_pwt variant has an intermediate value for the first phase, indicating a slower structural alteration as compared with S56N, making the first kinetic phase last longer. The additional layer formation of S56N most probably leads to an overestimation of the maximum value of adsorbed protein to the respective functionalized surface as presented in Table 4 for this variant. Because of this behavior, the following discussion regarding adsorbed mass and orientation is limited to the HCA II_pwt and A23C/L203Cox variants.

**Adsorption to the Negatively Charged Surface (Fig. 3A)** —The proteins are adsorbed in the largest quantities to the negatively charged surface. Despite this, the adsorbed mass does not come close to any of the theoretical coverage values for a close packed monolayer or a model based on the random sequential adsorption (Tables 4 and 5). However, we have recently been able to determine the adsorption orientation of HCA II in the native state to negatively charged silica nanoparticles by the use of fluorescent probes (44). This study revealed a strong pH dependence on the adsorption orientation and that at pH 8.5 the protein adsorbed in neither fully side-on nor fully end-on orientations, but rather in orientations that lead to an occupied surface area that are an approximate average of these two extremes. In terms of mass adsorbed, this would thus give a number that is the average between fully side-on and fully end-on (approx. 280 ng/cm² for a close packed monolayer and 150 ng/cm² for random sequential adsorption (RSA)). As is evident from the experimentally determined masses (Table 4) the adsorption to negatively charged surfaces comes very close to a theoretical value for adsorption in the native state, in a mixed end-on and side-on orientation in a random sequential mode (Table 5). This is also supported by the lack of an overshoot or lag phase in the adsorption isotherms, which indicates that no reorientation takes place during or after the adsorption step. The fact that the negatively charged surface is also the surface that displays the largest desorption upon washing with buffer further supports the notion that the protein (especially the A23C/L203Cox variant) is in its native state.

**Adsorption to the Hydrophilic Surface (Fig. 3B)** —The hydrophilic surface comes second regarding the amount of adsorbed protein. Also, the overall appearance of adsorption and desorption resembles very closely that of the negatively charged surface. The adsorbed protein mass is, in this case, close to the value for an RSA model with native protein adsorbing in a side-on manner (Table 4 and 5). Although we lack experimental data regarding the absolute orientation at hydrophilic surfaces, the large amount of desorbed protein upon washing from this surface also implies that the protein (A23C/L203Cox) has to a large extent remained in its native state, supporting side-on orientation adsorption.

**Adsorption to the Hydrophobic Surface (Fig. 3C)** —The adsorption at the hydrophobic surface is somewhat smaller than the adsorption at the hydrophilic surface. As it is known that hydrophobic surfaces are often denaturing, because of the tendency to force proteins to expose internal, hydrophobic residues, it is interesting to compare the adsorption with a theoretical monolayer based on the molten-globule state of the protein (Table 5). In this case the maximally adsorbed protein mass (Table 4) does in fact come fairly close to the theoretical RSA value for the protein adsorbing in a side-on manner that subsequently adopts a molten-globule state. This is especially evident by the HCA II_pwt variant which reaches the molten-globule state faster than the more stable A23C/L203Cox variant. Moreover, the substantially less protein desorption upon washing, indicates that the adsorbed proteins are more tightly bound to the hydrophobic surface.

**Adsorption to the Positively Charged Surface (Fig. 3D)** —The adsorption behavior of the proteins to the positively charged surface is significantly different as compared to that for the other surfaces, with a very low mass adsorbed, despite the fact that the protein is expected to carry a net negative charge at pH 8.5. However, this is most probably due to adoption of conformations that are even less ordered than the molten-globule state. This interpretation is also supported by the very low amplitude of the initial phase upon adsorption and the low
desorption of protein upon washing, with virtually no release of S56N and only a minor fraction of desorbing HCA II\textsubscript{pwt}. This indicates that the surface is highly denaturing, leading to a substantial increase of interactions between the surface and the protein with time. It is also noteworthy that adsorption isotherms of this surface show that none of the protein variants reaches a plateau value during the experimental time.

**Correlation Between Protein Stability and Desorption**—We have chosen not to make an attempt to analyze the desorption kinetics quantitatively since the desorption occurs from different types of interactions, for example, where the more stable A23C/L203C\textsubscript{ox} would desorb from the actual surface, whereas the less stable S56N variant would display desorption from a partial additional layer. The most striking feature of the four differently functionalized surfaces and the three stability variants is the difference in the amount of desorbed protein. Firstly, there is a strong correlation between how much protein is released upon washing with buffer and how much protein that can be maximally adsorbed to the surface, thus, the more denaturing the surface (low release of adsorbed protein) the lower is the adsorbed amount, which can be explained both in terms of faster denaturation rate of the protein at the surface and by the degree of denaturation. Secondly, as can be seen in Table 4 and Fig. 3, there is a very strong correlation between protein stability and amount of protein desorbed on all surfaces. The correlation between increased protein stability and increased desorption upon washing with buffer becomes even more evident when the percentage of desorbed protein is plotted as a function of protein stability, expressed as kcal/mol from chemical denaturation (16, 30) (Fig. 4A) or as T\textsubscript{m} (Fig. 4B). On the least denaturing surface (negatively charged) about 90 % of the stable A23C/L203C\textsubscript{ox} variant is desorbed by washing with buffer, whereas only 40 % of the least stable S56N variant is desorbed. On the most denaturing surface (positively charged), less than 10 % of the destabilized S56N variant is desorbed, whereas about half of the stabilized A23C/L203C\textsubscript{ox} variant is desorbed and the HCA II\textsubscript{pwt} is intermediate between the two. This pattern is evident, from the least to the most denaturing surface.

**CONCLUSIONS**

The negatively charged and the hydrophilic surfaces are the ones that are the least denaturing of the four surfaces tested. There are also indications that the adsorption to the surfaces occurs according to a random sequential adsorption (RSA) model with specific orientations, with the negatively charged surface adsorbing mainly native protein in a mixed side-on and end-on manner, in agreement with earlier findings (Karlsson and Carlsson, 2005), whereas the hydrophilic surface adsorbs in a side-on manner. The more denaturing, hydrophobic surface, has a lower maximal surface load as the protein is likely to adopt a more expanded structure on this surface as compared to less denaturing surfaces. Furthermore, the adsorbed mass is, in this case, close to the theoretical value for the protein being in the molten-globule state that has adsorbed in a side-on orientation under random sequential adsorption. The adsorption to the positively charged surface is more difficult to interpret, as both the adsorbed and desorbed mass is very low. It is evident from Fig. 3 and Table 4 that the stability of the protein does not influence the amount of adsorbed protein such that a more stable protein will adsorb to a less extent than a destabilized protein. On the contrary, the more stable A23C/L203C\textsubscript{ox} actually adsorbs with the highest surface load on several of the surfaces. The interpretation of these findings can be explained in terms of the denaturing behavior of the three protein variants, where we have earlier shown that S56N is completely converted to a molten-globule form within about 15 min, when adsorbed to negatively charged silica nanoparticles, whereas HCA II\textsubscript{pwt} only slowly goes through this transformation. These different behaviors of the three protein stability variants do not only influence the total amount of adsorbed protein, but also the kinetics of adsorption.

Most interestingly, the most stable protein variant, A23C/L203C\textsubscript{ox}, is the one that most readily desorbs from the various surfaces,
despite being the protein that adsorbs to the largest extent. To conclude, this also emphasizes the importance of having a well-defined system when comparing the behavior of different proteins at surfaces, as only a single parameter, in this case stability, gives such significant differences in adsorption/desorption behavior. Furthermore, it is only by knowing the stability and unfolding behavior of the protein variants and by monitoring both the adsorption and desorption characteristics that we have been able to draw conclusions about the state and orientation of the adsorbed proteins.

Finally, our results also demonstrate that using protein engineering to increase protein stability with the aim to reduce irreversible protein adsorption can be added to the list of rationales for protein stabilization.

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**FOOTNOTES**

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1The abbreviations used are: A23C/L203C<sub>ox</sub>, HCA II<sub>pwt</sub> with a Ala23→Cys and a Leu203→Cys mutation in the oxidized state; HCA II<sub>pwt</sub>, pseudo wild-type of human carbonic anhydrase II with a Cys206→Ser mutation; RSA, Random Sequential Adsorption; SAM, Self-Assembled Monolayer; SPR, Surface Plasmon Resonance; S56N, HCA II<sub>pwt</sub> with a Ser56→Asn mutation.
**FIGURE LEGENDS**

**Fig. 1.** Protein stability curves for HCA II variants, based on values obtained by tryptophan fluorescence measurements in various concentration of GuHCl. Symbols: (○) S56N; (●) HCA II pwt; (Δ) A23C/L203Cox. The HCA II pwt and S56N curves were fitted to a three-state transition (N→I→U), and the A23C/L203Cox was fitted to a two-state function (N→U). Data for HCA II pwt and S56N was taken from Ref. 16 and for A23C/L203C from Ref. 30. The parameters monitored are summarized in Table 1.

**Fig. 2.** Infrared reflection-absorption spectra of the methyl-, amino- hydroxy- and sulphate-terminated thiols assembled on gold. The region shown is the fingerprint region of the spectra.

**Fig. 3A-D.** Isotherms of the adsorption and desorption of protein variants. (A) negatively charged surface, (B) hydrophilic surface, (C) hydrophobic surface, (D) positively charged surface. Symbols: S56N (cyan); HCA II pwt (black) and A23C/L203Cox (red). The horizontal bars denote the amplitude of the first kinetic phase of the respective protein variant (equivalent to the values in Table 3). Each isotherm is the average of duplicate or triplicate SPR measurements.

**Fig. 4A-B.** Percentage of desorbed protein after washing with buffer for 30 min plotted against the (A) thermodynamic stability and (B) melting point (T_m) of investigated protein variants for each surface. Symbols: (▼) negative surface, (○) hydrophilic surface, (●) hydrophobic surface and (+) positive surface.
Table 1. Characteristics of the protein variants

| Protein          | Fractional surface accessibility<sup>a</sup> | Position | C<sub>m, N</sub><sup>b</sup> (M) | C<sub>m, I</sub><sup>b</sup> (M) | C<sub>m, U</sub><sup>b</sup> (M) | Stability (kcal/mol) | T<sub>m</sub> (°C) | CO₂-hydration activity (%) |
|------------------|-----------------------------------------------|----------|-------------------------------|-------------------------------|-------------------------------|----------------------|-----------------|-----------------------------|
| S56N<sup>c</sup>  | 0                                             | β-strand 2 | 0.4 M                         | 1.8 M                         | 2.9<sup>d</sup>               | 46<sup>d</sup>      | 81              |                             |
| HCA II<sub>pwt</sub><sup>c</sup> | 0.01                                          | β-strand 7 | 1.0 M                         | 1.9 M                         | 7.9<sup>d</sup>               | 59<sup>d</sup>      | 100             |                             |
| A23C/L203C<sub>ox</sub><sup>e</sup> | 0.08/0                                        | 3<sub>10</sub>⁺ helix/turn | 1.8 M                         |                               | 11.0<sup>f</sup>             | 74<sup>f</sup>      | 55              |                             |

<sup>a</sup> The fractional accessibility of the wild-type amino acids to be replaced.

<sup>b</sup> C<sub>m, N</sub>, C<sub>m, I</sub>, and C<sub>m, U</sub> represent the transition midpoint concentrations from the native (N) to the intermediate (I) state, from the intermediate (I) to the unfolded state (U), and from the native (N) to the unfolded (U) state upon denaturation in increasing concentrations of guanidinium chloride, respectively.

<sup>c</sup> All data except melting point from ref. 16.

<sup>d</sup> For the N ⇔ I transition.

<sup>e</sup> All data except melting point from ref. 30.

<sup>f</sup> For the N ⇔ U transition.
### Table 2. Surface thickness\(^a\) and contact angles\(^b\)

| Surface functionality | d (Å)\(^c\)  | d (Å) estimated\(^d\)  | θ\(^e\),\(w\)\(^g\) | θ\(^e\),\(h\)\(^d\) | θ\(^f\),\(w\)\(^g\) | θ\(^f\),\(h\)\(^d\) |
|-----------------------|--------------|------------------------|---------------------|---------------------|---------------------|---------------------|
| Negative (-SO\(_4^–\)) | 11.3 ± 1.0 (7) | 11.4 | <10 | <10 | <10 | <10 |
| Hydrophilic (-OH)\(^j\) | 9.0 ± 0.4 (5) | 9.1 | 26 ± 5 | <10 | <10 | <10 |
| Hydrophobic (-CH\(_3\)) | 9.1 ± 0.8 (7) | 8.5 | 82 ± 1 | 24 ± 1 | 66 ± 1 | <10 |
| Positive (-NH\(_3^+\)) | 9.3 ± 0.9 (7) | 9.1 | 37 ± 1 | <10 | <10 | <10 |

\(^a\) Measured by ellipsometry.
\(^b\) Measured by goniometry.
\(^c\) Values are given with a 95% confidence interval. Number of measurements is given in parenthesis.
\(^d\) From molecular modeling.
\(^e\) Contact angles. Values are given with a 95% confidence interval, measured on 5 separate samples.
\(^f\) Advancing contact angle.
\(^g\) Water.
\(^h\) Hexadecane.
\(^i\) Retracting contact angle.
\(^j\) Values presented are from previous work (26).
Table 3. Kinetic data associated with the initial adsorption step.

| Surface    | S56N |           | HCA II_{pmt} | A23C/L203C_{ox} |
|------------|------|-----------|--------------|-----------------|
|            | $k_1$ (min$^{-1}$)$^a$ | $A_1$$^a$ | $r^2$$^b$    | $k_1$ (min$^{-1}$)$^a$ | $A_1$$^a$ | $r^2$$^b$ | $k_1$ (min$^{-1}$)$^a$ | $A_1$$^a$ | $r^2$$^b$ |
| Negative   | 28   | 82        | 0.997        | 22              | 91          | 0.996      | 18              | 141         | 0.992     |
| Hydrophilic| 34   | 66        | 0.995        | 39              | 70          | 0.987      | 25              | 81          | 0.997     |
| Hydrophobic| 37   | 45        | 0.999        | 30              | 54          | 0.994      | 29              | 62          | 0.999     |
| Positive   | 33   | 6         | 0.999        | 36              | 12          | 0.999      | 16              | 22          | 0.999     |

$^a$ The rate constants and amplitudes were calculated using a nonlinear fit program (see Material and Methods). $^b$ The goodness of fit was calculated by the $r^2$ coefficient of determination.
Table 4. Adsorbed protein mass as measured with SPR immediately before and after the washing step.

| Surface   | S56N                      | HCA II<sub>pwt</sub> | A23C/L203C<sub>ox</sub> |
|-----------|---------------------------|-----------------------|---------------------------|
|           | Max. adsorbed (ng/cm<sup>2</sup>)<sup>b</sup> | After washing (ng/cm<sup>2</sup>)<sup>b</sup> | % desorbed | Max. adsorbed (ng/cm<sup>2</sup>)<sup>b</sup> | After washing (ng/cm<sup>2</sup>)<sup>b</sup> | % desorbed | Max. adsorbed (ng/cm<sup>2</sup>)<sup>b</sup> | After washing (ng/cm<sup>2</sup>)<sup>b</sup> | % desorbed |
| Negative  | 165± 3.9                  | 99± 2.0               | 40 | 145± 0.0                  | 35± 0.0               | 76 | 161± 0.7                  | 20± 2.3               | 88 |
| Hydrophilic | 111± 0.0                 | 72± 0.0               | 35 | 102± 0.0                  | 33± 0.0               | 68 | 113± 3.0                  | 24± 4.5               | 79 |
| Hydrophobic | 108± 4.5                 | 92± 4.1               | 15 | 91± 2.0                   | 55± 3.0               | 40 | 102± 9.5                  | 48± 0.7               | 53 |
| Positive  | 64± 1.5                   | 59± 1.0               | 8  | 53± 4.1                   | 34± 4.0               | 36 | 71± 1.8                   | 37± 3.2               | 48 |

<sup>a</sup> The washing step with buffer was initiated after a 13 min adsorption step and was finished after 30 min.

<sup>b</sup> Values are the mean of duplicate or triplicate SPR measurements, ± is the standard error of the mean.
Table 5. Theoretically adsorbed masses

| State and orientation               | Size of protein (Å) | Surface contact area (nm²) | Theoretical close packed monolayer coverage (ng/cm²) | Theoretical monolayer coverage (RSA) (ng/cm²) |
|------------------------------------|---------------------|----------------------------|---------------------------------------------------|-----------------------------------------------|
| HCA II native side-on              | 55 x 42 x 39        | 23.8                       | 205                                               | 112                                           |
| HCA II native end-on               | 42 x 39             | 13.8                       | 351                                               | 192                                           |
| HCA II molten globule side-on      | 63 x 46 x 43        | 31.2                       | 156                                               | 85                                            |
| HCA II molten globule end-on       | 46 x 43             | 16.6                       | 293                                               | 160                                           |
Fig. 1

Fractional change

[Gu-HCl] (M)

0 0.2 0.4 0.6 0.8 1

0 1 2 3 4

[Gu-HCl] (M)
Fig. 2

\[ \text{X} = \text{CH}_3, \text{OH}, \text{SO}_4^- \text{ or } \text{NH}_3^+ \]
Fig. 3
Fig. 4
Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability
Martin Karlsson, Johan Ekeroth, Hans Elwing and Uno Carlsson

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