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High-performance liquid chromatography of amino acids, peptides and proteins

CXXI.* 8-Hydroxyquinoline–metal chelate chromatographic support: an additional mode of selectivity in immobilized-metal affinity chromatography

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ABSTRACT

In our associated studies, the binding of proteins in immobilized-metal affinity chromatography has been shown to be independent of surface-exposed histidines and aromatic amino acid residues, when hard metals are used. The present investigation documents the behaviour of iminodiacetic acid (IDA) and 8-hydroxyquinoline (8-HQ) immobilized on Sepharose CL-4B and chelated with detergent (Brij-35), at pH 7.0. The 8-HQ gel had a higher capacity for tuna heart cytochrome *c* (THCC) when Fe^{3+} was immobilized than when Al^{3+} or Yb^{3+} was used, whilst 8-HQ-Cu^{2+} and 8-HQ-Ca^{2+} did not bind this protein. The equivalent IDA chelates showed no binding of the protein. The THCC was recovered from the 8-HQ-Fe^{3+}, -Yb^{3+} and -Al^{3+} supports upon elution with high concentrations of phosphate, glutamate or malonic acids, suggesting that acidic amino acid residues were involved in the binding. Application of molecular graphics procedures reveals that the 8-HQ-metal^{3+} chelate represents a new class of coordination geometry for binding to proteins, and hence offers an additional mode of selectivity in immobilized-metal affinity chromatographic separations.

INTRODUCTION

Immobilized metal affinity chromatography (IMAC) has been a relatively recent addition to the many modes of protein chromatography. Its high degree of specificity, as was first suggested by Everson and Parker for metalloproteins [1] and significantly advanced by Porath *et al.* [2], Sulkowski [3] and other investigators with globular proteins [4–7], has seen this technique become one of the more popular chromatographic modes.

Borderline type metals, as defined by Pearson [8], such as Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}, have been the most commonly used in IMAC for protein purification. Their preference for nitrogen-containing amino acid side chains has been exploited by many workers [9–11]. The outcome has been the observation that when these metals are used, histidine is the primary putative electron donor, and hence represents an important interactive amino acid, accessible on the surface of proteins. Tryptophan and cysteine play a secondary role in the protein binding, depending on the presence of histidine [12]. Although most amino acids are able to interact individually with immobilized metal chelating systems [11], most do not seem to do so when exposed on the surface of a protein. This degree of specificity for histidine, by borderline metals, has been recently highlighted by the cloning of protein products with polyhistidine tails for later purification using

* For part CXX, see ref. 41.

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IMAC [13], the resolution of similar peptides varying by none, one, two, and three histidines [14], and the use of IMAC as a facile probe for the detection of histidine residues on proteins [15].

Using species variants of cytochrome *c* as model proteins, Hemdan *et al.* [15] were able to differentiate between cytochrome *c* variants containing none, one and two histidine residues on an iminodiacetic acid (IDA)-Cu^{2+} column. These investigators did not observe any binding of tuna heart cytochrome *c* (THCC) when this protein was loaded in phosphate buffer at high ionic strength. The THCC does not have any exposed histidine, tryptophan or cysteine residues.

Hard metals have not been extensively studied in IMAC. Fe^{3+} and Ca^{2+} have received some attention [16–18] whilst the use of hard metals such as the lanthanides and aluminium have rarely been reported [19,20]. It has emerged that hard metal IMAC differs somewhat from conventional IMAC in that a number of different modes of interactions are involved, including composite electrostatic and coordination type bonding. Sulkowski [17] has suggested that electrostatic interactions are more evident for basic proteins in hard metal IMAC. Ramadan and Porath [21] indicated that aspartate and glutamate showed a higher affinity for Fe^{3+} than did other amino acids, including histidine and tryptophan. Cysteine had the highest affinity for Fe^{3+} of all the common protein amino acids. However, Fe^{3+} has also shown a high degree of affinity toward phosphoproteins and glycoproteins [17,22, 23].

In the present investigation, THCC was chosen as a model protein for the study of its interaction with the hard metals, Fe^{3+}, Al^{3+}, Yb^{3+}, Ca^{2+} and Cu^{2+} as a borderline metal ion control. THCC has a large number of surface exposed aspartic and glutamic acid residues [24] and we sought to exploit the preference of hard metals for oxygen rich groups. These metals were immobilized on covalently bound IDA and 8-hydroxyquinoline (8-HQ) chromatographic supports (Fig. 1). The former chelating support has been extensively used in IMAC and behaves as a tridentate chelator [3]. To our knowledge the 8-HQ ligand has only been used once before, with Zn^{2+} as the immobilized metal, for the isolation of metalloproteins [1], although it has been well characterized on silica supports [25,26]

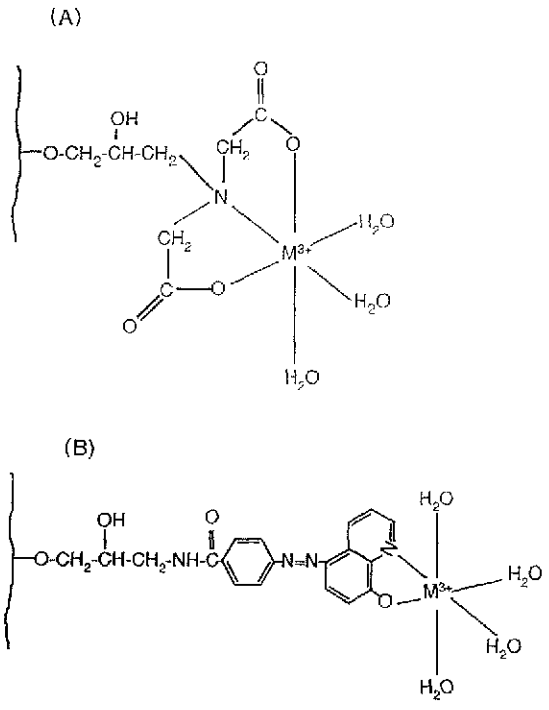

Fig. 1. Diagrammatic representation of the immobilized tridentate chelating agent IDA (A) and the bidentate chelating agent 8-HQ (B) on Sepharose-CL-4B. M = Metal.

and methacrylate supports [27,28], where it has been used extensively for the chromatography of metal ions [29]. Studies on the chromatography of metal ions on immobilized 8-HQ, in a variety of conditions has shown good selectivity for the metals used in the present work [30–32]. The 8-HQ ligand is a bidentate chelating agent but its rigid structure attributes it with higher stability constants for the hard metals compared to IDA [33]. This property is advantageous in protein chromatography since it allows an extra site on the metal, and thus enables an additional mode of selectivity to be achieved. Because of the high value of the stability constants metal leakage is minimized and thus the effective capacity of the immobilized metal complex for the protein is increased.

We report for the first time the use of hard metals immobilized on 8-HQ-Sepharose CL-4B for the isolation of proteins. The use of this adsorbent enables the isolation of THCC using IMAC methods which hitherto have not been applicable to this protein. Furthermore, the results indicate that binding of

THCC and other cytochromes *c* to hard metals, immobilized on 8-HQ-Sepharose CL-4B, is independent of histidine, tryptophan, and cysteine residues in the protein. Application of the 8-HQ ligand thus introduces an additional mode of selectivity in IMAC separations.

EXPERIMENTAL

Materials

Metal salts and 8-HQ were purchased from Aldrich (Milwaukee, WI, USA). THCC type XI, and iminodiacetic acid were purchased from Sigma (St. Louis, MO, USA). Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). All other reagents were of analytical-grade purity and Milli-Q water was used for all preparations. All glassware was extensively washed with dilute nitric acid before use.

Immobilization of chelating agents

The 8-HQ-Sepharose-CL-4B was prepared by modification of the procedure by Hill [34]. In brief, 50 g of Sepharose-CL-4B were epoxy activated using 10 ml of epichlorohydrin in 25 ml of 2 *M* NaOH and 94 mg of $NaBH_4$. The reaction mixture was gently stirred for 15 h at room temperature and then washed under suction with 20 volumes of Milli-Q water. After washing, 1.5 volumes of 25% (v/v) ammonia solution were added to the suctioned dry gel and incubated in a shaking water bath at 40°C, for 1.5 h. The aminated gel was subsequently washed with 10 volumes of Milli-Q water, before passage via acetone, into a neat chloroform solvent using 5 volumes of 20% (v/v) solvent increments. The aminated gel in neat chloroform, was benzoylated with 5 g of *p*-nitrobenzoyl chloride and 5 ml of triethylamine. This mixture was incubated at 50°C for 48 h in a shaking water bath. The gel was extracted out of the chloroform via acetone and into Milli-Q water using 5 volumes of 20% (v/v) solvent decrements. The gel was checked for free amines using the trinitrobenzene sulphonic acid test [34]. If free amines were observed, the incubation was continued. The gel was then placed into a 100 ml solution of 5% (w/v) sodium dithionite, to reduce the $-NO_2$ groups to $-NH_2$, and incubated at 45°C, for approximately 15 h in a shaking water bath. The gel was then washed with 20 volumes of Milli-Q water and incubated at 0–4°C, for 30 min with 100 ml of 2% (w/v) sodium nitrite in 10 m*M* HCl whilst gently stirring. At this stage the gel turned yellow indicating that the diazo bond had formed. The gel was then washed with 10 volumes of cold Milli-Q water and passaged into neat ethanol solution using 5 volumes of 20% (v/v) increments of solvent. The final mixture contained the gel in 100 ml of neat ethanol and 2% (w/v) 8-HQ. The mixture was gently stirred for 3 h at room temperature. After the incubation, the gel had turned a deep, "fire red" colour, indicating that the 8-HQ was immobilized. The gel was then suspended in a final 20% (v/v) ethanol solution where it was stored at 4°C until used for chromatography. Nitrogen analysis done by Dairy Technical Services (Melbourne, Australia), revealed that a substitution of $518 \cdot 10^{-6}$ mol of 8-HQ/g dry gel was obtained.

IDA-Sepharose-CL-4B was prepared without deviation by the method of Porath and Olin [35]. Nitrogen analysis by Dairy Technical Services, revealed $500 \cdot 10^{-6}$ mol of IDA/g dry gel.

Loading of metals

Various studies have shown that metal nitrates or sulphates were preferred over the less dissociating chloride equivalents [36], so as to avoid the immobilization of metal chloride species. The chelate gels, prepared as described above, were incubated at room temperature with either 10 m*M* ferric nitrate or aluminium nitrate, or 50 m*M* calcium nitrate, copper nitrate or ytterbium sulphate, in the presence of 0.1 *M* KNO_3, whilst stirring under reduced pressure (*ca.* 20 mmHg) for 30 minutes to ensure the gel pores were filled with liquid. The lower concentration of metal was used for iron and aluminium to minimize the formation of hydrolytic species which develop more readily with these metals.

The gel was then incubated with 50 m*M* acetic acid and 0.1 *M* KNO_3 pH 4.0 in ratio of 1:10, for 10 min under vacuum to remove any lossely bound ions.

Chromatography

The metal-bound gel was then washed with the equilibration buffer, 20 m*M* imidazole in 0.5 *M* NaCl and 0.005% (v/v) Brij-35 pH 7.0, and incubated in this buffer, under vacuum for 30 min. The

equilibrated gel was then packed into 20-ml Bio-Rad econocolumns.

THCC was resuspended in equilibrating buffer to a concentration of 1 mg/ml. Aliquots of 0.2 ml were loaded on to the metal columns and washed with 5 ml of equilibration buffer. The breakthrough/wash volume was collected and labelled the non-adsorbed fraction. Then 5 ml of elution buffer, either 20 m*M* imidazole and 0.16 *M* K_2HPO_4 and 0.005% (v/v) Brij-35, pH 7.0 (buffer A), 20 m*M* imidazole and 0.16 *M* malonic acid and 0.005% (v/v) Brij-35, pH 7.0 (buffer B), or 20 m*M* imidazole, 0.16 *M* sodium glutamate, 0.34 *M* NaCl and 0.005% Brij-35 pH 7.0 (buffer C), were then loaded onto the columns. This volume was collected and labelled the eluted fraction.

Quantitation of THCC was achieved by analytical high-performance liquid chromatography (HPLC) of the appropriate sample down a J. T. Baker C_{18} or C_8 reversed-phase column (250 × 4.6 mm I.D., 5 μm) using a 1 ml/min flow-rate and a linear gradient of 0.1% (v/v) trifluoroacetic acid to 0.09% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile over 30 min. The HPLC system used was a Hewlett-Packard HP 1090. The peak area was monitored using a diode array detector set at 215 nm and 400 mm. The sample areas were compared to those of an accurately weighed sample of THCC standard. The lower sensitivity level of this method was 75 ng.

Computer analysis of crystallographic structure

The Insight II programme from Biosym (San Diego, CA, USA) was used on an Iris molecular graphics terminal to determine the space filling model and the ribbon format model of THCC. The programme used crystallographic coordinates from the Brookhaven Data Bank for THCC (1CYT).

RESULTS

Binding behaviour of THCC on immobilized 8-HQ and IDA chelated with metal

Results are shown in Table I for the binding and elution behaviour of THCC on the tridentate IDA support chelated with Cu^{2+}, Ca^{2+}, Yb^{3+}, Fe^{3+} and Al^{3+}. THCC did not bind to any of the immobilized metals with the IDA support with the equilibration conditions used. There was no suggestion of detectable non-specific binding on this support as is indicated by the total recovery of THCC in all the non-adsorbed fractions.

TABLE I

BINDING BEHAVIOUR OF THCC ON IMMOBILIZED 8-HQ AND IDA CHELATED WITH METAL

THCC was resuspended in the equilibration buffer. 20 m*M* imidazole–HCl in 0.5 *M* NaCl and 0.005% (v/v) Brij-35 pH 7 before loading on to the 8-HQ and IDA supports as described under *Chromatography* in the Experimental section. THCC was quantitated as described under *Chromatography* in the Experimental section.

IMAC gels	Protein non-adsorbed (%)	IMAC gels	Protein non-adsorbed (%)
IDA-Cu^{2+}	>99	8-HQ-Cu^{2+}	88
IDA-Ca^{2+}	>99	8-HQ-Ca^{2+}	88
IDA-Yb^{3+}	>99	8-HQ-Yb^{3+}	60
IDA-Fe^{3+}	>99	8-HQ-Fe^{3+}	<1
IDA-Al^{3+}	>99	8-HQ-Al^{3+}	80
IDA-Blank	>99	8-HQ-Blank	85

Results are also shown in Table I for the binding behaviour of THCC on the bidentate 8-HQ support chelated with Cu^{2+}, Ca^{2+}, Yb^{3+}, Fe^{3+} and Al^{3+}. In this case, THCC bound to metal in the following quantitative order; $Fe^{3+} > Yb^{3+} > Al^{3+} > Ca^{2+}$, Cu^{2+}. Interestingly, up to 15% of THCC bound to the naked 8-HQ-Cl-4B support even though Brij-35 was included. More Brij-35 was not added so as to avoid exceeding the critical micelle concentration of this detergent. THCC binding to Cu^{2+} or Ca^{2+} immobilized to 8-HQ affinity gels was about the same as for the blank 8-HQ gel and thus these IMAC sorbents were considered not to have bound THCC specifically.

Elution behaviour of THCC on immobilized 8-HQ chelated with metal

Elution of the THCC from immobilized Fe^{3+}, Yb^{3+} and Al^{3+} was attempted with glutamic acid, malonic acid and phosphate (Table II). Glutamic acid and phosphate eluted approximately the same amount of bound THCC for the three metals tested. Not all the THCC however, could be accounted for in the eluted fractions. For example, only 70% of THCC could be accounted for in the case of Yb^{3+}, allowing for the 15% of THCC bound non-specifi-

TABLE II

ELUTION BEHAVIOUR OF THCC ON IMMOBILIZED 8-HQ CHELATED WITH METAL

Binding conditions for THCC on the 8-HQ metal support was as described for Table I. Elution of THCC was either by 20 m*M* imidazole and 0.16 *M* K_2HPO_4 and 0.005% (v/v) Brij-35 pH 7 (buffer A), 20 m*M* imidazole and 0.16 *M* malonic acid and 0.005% Brij-35 pH 7 (buffer B), or 20 m*M* imidazole, 0.16 *M* sodium glutamate, 0.34 *M* NaCl and 0.005% (v/v) Brij-35 pH 7 (buffer C). THCC was quantitated as described under *Chromatography* in the Experimental section.

IMAC gels	Percentage of bound protein eluted		
	Buffer A	Buffer B	Buffer C
8-HQ-Yb^{3+}	58	35	52
8-HQ-Fe^{3+}	31	9	35
8-HQ-Al^{3+}	13	<1	15

cally. In the case of immobilized Al^{3+}, nearly 70% of bound THCC could not be eluted. Malonic acid appears to be an even less effective eluting agent than does glutamic acid or phosphate. A pattern for recovery of THCC also emerges from these results. Recovery of THCC is greatest for Yb^{3+} followed by Fe^{3+} and then Al^{3+}, independent of elution conditions.

Computer analysis of THCC

Examination of the molecular surface of THCC on the Iris revealed the accessible amino acid residues that could donate electrons and hence afford binding to the immobilized metals. The amino acids were 3 aspartates, 5 glutamates, 2 asparagines, 4 glutamines and 16 lysines. The lysine residues are probably not involved since their ε-amino groups are still fully protonated at pH 7.0 and, with the exception of Cu^{2+}, the other metals being hard Lewis acids, would not have any strong affinity for amino acid side chain groups, especially in the presence of a large molar excess of imidazole. The amino acids that are therefore likely to bind and are accessible to solvents as suggested by the Insight II programme and the available literature [37], are aspartate 50, aspartate 62, aspartate 2, aspartate 93, glutamate 44, glutamate 69, glutamate 66, glutamate 21, and glutamate 90. Fig. 2 shows the ribbon form of THCC, with glutamate 69 and 66, and aspartate 62, in close proximity to each other, and at

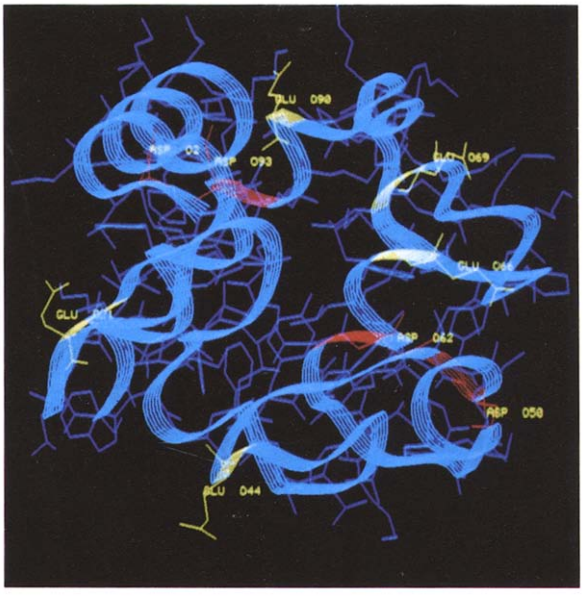

Fig. 2. Ribbon print of THCC depicting the surface accessible glutamate (yellow shade) and aspartate (red shade) residues on the bends of helices reproduced from the Insight II programme.

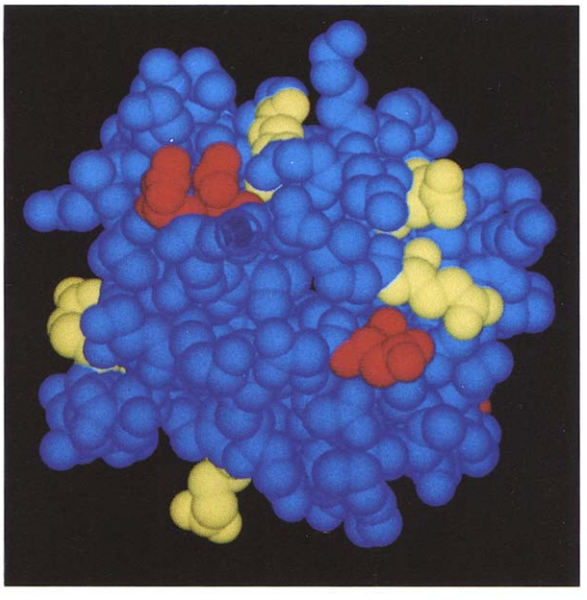

Fig. 3. Space filling model of THCC depicting the surface exposed glutamates (yellow) and aspartates (red) residues grouped into two clusters. THCC in this figure is in the same orientation as that depicted in the ribbon form of Fig. 2.

bends of a helix. Furthermore, aspartate 2 and 93, and glutamate 90 also appear to form a negatively charged cluster. Glutamate 21, and 44 and aspartate 50 all appear solvent accessible but with no vicinal negatively charged groups. The two negatively charged clusters are well separated form each other as is shown in the space filling diagram of Fig. 3.

DISCUSSION

Since the inception of using immobilized metals in IMAC for the isolation of proteins, most of the work has used borderline type Lewis metals, immobilized to the tridentate chelating agent IDA. Other chelating agents that have been immobilized for use in IMAC, include ethylenediaminetetraacetic acid [38], tris(carboxymethyl)ethylenediamine (TED) [35], the carboxymethylated agents [18,13,39] and the monohydroxamate agent [21]. With the exception of the latter ligand structure, all the above chelating agents are tri-, tetra- and pentadentate. As documented here, we have successfully expanded this range by using a bidentate ligand, namely 8-HQ, used for the first time in IMAC of proteins in combination with hard metal ions. In view of the current interest in exploiting the specificity of IMAC as a probe to study the surfaces of proteins, we have also examined the possibility of amino acids in a tertiary protein context, other than histidine, tryptophan and cysteine that may also have an affinity for metals. Using THCC which does not have any of these amino acids on its surface, in association with hard metals immobilized on 8-HQ-Sepharose CL-4B, we have shown that binding of proteins in IMAC, can be independent of histidine, tryptophan and cysteine.

Hemdan *et al.* [15] were unable to isolate THCC on an IDA-Cu^{2+} IMAC column at pH 7.0 using a phosphate buffer, even though THCC has 16 lysines which are solvent accessible. These workers attributed this to a lack of histidines on the surface of the protein. Our results indicate however, that a change from phosphate to an imidazole buffer at pH 7.0 still did not ellicit any binding of THCC to the IDA-Cu^{2+} or 8-HQ-Cu^{2+} sorbents. This observation is consistent with the view that borderline metals, such as Cu^{2+} have a low preference for oxygen-rich groups. Furthermore, it can be concluded that Cu^{2+} has no (or very low) affinity for any other exposed electron-donating groups on THCC eventhough this M^{2+} ion has had an extra coordination site made available by its immobilization on to 8-HQ-Sepharose Cl-4B.

By immobilizing hard metals on 8-HQ-Sepharose CL-4B we were able to retain and subsequently elute THCC. These results can be explained by the extra metal coordination site made available by their immobilization on the bidentate 8-HQ. IDA-Ca^{2+} and 8-HQ-Ca^{2+} did not bind any THCC, eventhough it is considered a hard metal. Further work is currently being done to extend these observations.

Our results suggest that specific THCC binding on 8-HQ immobilized metals is through aspartic and/or glutamic acid residues rather than nitrogen rich groups such as lysines or hydrophobic residues. The behaviour of horse heart and dog heart cytochrome *c* (two variants with surface histidine residues and with similar surface distribution of lysines and hydrophobic amino acid residues) with hard metals immobilized on IDA and 8-HQ in the presence of imidazole buffer further support these findings [40].

The bound THCC could be recovered in varying yield form the metal supports without changing the ionic strength or the pH of the elution buffer from that of the gel equilibration buffer. Of the eluents examined (Table II), malonic acid was the least effective in eluting THCC from the immobilized metals. This observation can be explained by the low affinity of metal ions for malonic acid relative to glutamate and phosphate [33]. The THCC that was not recovered using these elution conditions may represent cytochrome molecules which are bound at higher affinity sites involving clusters of aspartate and glutamate residues as shown in Figs. 2 and 3. In conjunction with this, the heterogenous nature of the spacer arm and ligand may also be contributing to the lack of total THCC recovery. Our proposal that aspartate and glutamate are directly responsible for the binding of THCC to hard metals immobilized on 8-HQ sorbents is the subject of further investigation.

The preliminary results presented in this paper introduce an additional mode of bidentate interaction in IMAC. Furthermore, these data suggest that the use of hard metal ions in IMAC may have important attributes for the study of the surface interactions and properties of proteins.

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