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Protection of enzymes by aromatic sulfonates from inactivation by acid and elevated temperatures

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Abstract

Selected azoaromatic sulfonate anions protect enzymes from inactivation by acid and elevated temperatures. These anionic sulfonate ligands bind to enzyme molecules by forming ion pairs between negatively charged sulfonate groups and positively charged protein groups as demonstrated by the binding stoichiometry determined using isothermal titration calorimetry. When the number of bound sulfonate anions is equal to the total positive charge of the protein, the protein–ligand complexes coprecipitate. Coprecipitation and protection are well correlated, but coprecipitation does not always result in protection. The coprecipitation–protection reactions are reversible. Ligand anions can be removed with anion exchange resins, and full enzymatic activity recovered. Comparison of 29 azoaromatic sulfonate ligands showed that small structural differences in the ligands produce large differences in their abilities to protect enzymes. Some protected enzymes were up to 1000 times more resistant to acid-inactivation, and their inactivation temperatures were over 10°C higher compared to nonprotected enzymes. Protection of six sulfhydryl proteases, namely papain, actinidin, chymopapain, bromelain, papaya protease omega, and ficin were compared. These proteases are highly homologous, have almost identical polypeptide chain fold, but differ in the numbers and locations of positive charges, which were crucial factors determining protection. Catalase enzyme, which is larger than papain and of a different class, was also protected by sulfonate ligands from inactivation by acid. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Protein protection by ligands; Protein coprecipitation; Papain family sulfhydryl proteases; Catalase; Azobenzene sulfonate ligands; Ligand binding to proteins by forming ion pairs; 1-Anilino-8-naphthalene sulfonate; Isothermal titration calorimetry

1. Introduction

In enzyme isolation and purification, damage to enzymes by acidic conditions and elevated temperatures often incur severe losses of activity. It is long known that naturally occurring substrate molecules, cofactors and other bioactive compounds frequently are good protective agents for their respective enzymes [4]. Synthetic compounds foreign to an enzyme's biofunction, are not usually expected to be protective. Nevertheless, synthetic azoaromatic sulfonate dye anions frequently provide notable protection for enzymes from inactivation by acid [15].

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Synthetic azoaromatic sulfonate ligands, including sulfonate ligands used for other purposes such as 1-anilino-8-naphthalene sulfonate (ANS), initially bind to proteins by forming ion pairs between ligand sulfonate groups and the positively charged sites of protein molecules [14]. The primary binding forces are of electrostatic nature. However, the protective ability of these ligands sharply depends on the structure of the nonpolar azoaromatic group. This phenomenon is the main topic of this paper.

Conventionally, precipitation of proteins in strongly acidic solution is equated to protein denaturation. However, coprecipitation of enzymes with optimal synthetic ligands may indeed be protective. The enzymes may be inhibited while in liganded, coprecipitated state. However, these coprecipitation–protection reactions are usually fully reversible. Ligand anions can be quickly removed with an anionic exchange resin. Fully active enzymes thereby are released into solution. Such ligands were named 'Matrix Coprecipitating and Cocrystallizing Ligands' (MCC ligands). Their ability to cocrystallize with proteins and cationic amino acids was discussed earlier [5]. Coprecipitative capability of MCC ligands makes them interesting agents in protein isolation and purification from crude and dilute sources [24]. Protective capability of MCC ligands further expands their usefulness in protein separations. Protein protection enables use of strong acid during protein isolation and purification. Protective ability of MCC ligands against temperature inactivation enables use of elevated temperatures during protein isolation and purification process.

A family of sulfhydryl proteases from plants (papain, bromelain, ficin, actinidin, chymopapain, and papaya protease omega) [1–3] was chosen for this study for the following reasons. First, these proteases can be isolated and purified in large scale for biophysical studies. Second, their sequences are highly homologous. Their crystallographic structures are solved to atomic resolution (except for bromelain and ficin), showing that the fold of the polypeptide chain in each enzyme is very similar. Third, total numbers of charged amino acids in each protease are similar, but ratios of positively to negatively charged amino acid numbers vary considerably, producing isoelectric points in this enzyme family ranging from 3 to 11. Fourth, these proteases do not refold after being denatured by acid or elevated temperatures. They do not regain enzymatic activity after removal of such stress unless protected. Sulfhydryl proteases used in this work already lack the propeptide sequence which is necessary for the correct folding of the enzyme molecule. The propeptide sequence is clipped off during protein processing in plants [12,23]. This property enables monitoring protein protection by measurement of enzymatic activity after relief of acidic stress and ligand removal without possibility of protein renaturation.

In addition to the plant sulfhydryl proteases, data is presented below for mammalian catalase protection from acidic inactivation. The catalase molecule is about 10 times larger in MW than papain, and has a very different structure. However, catalase is also protected by an aromatic sulfonate ligand that protects bromelain.

2. Materials and methods

2.1. Sulfhydryl proteases of the papain family

Papain, bromelain, and ficin were purchased from Sigma. Another bromelain sample was purchased from EY-labs. Chymopapain and protease omega (caricain, papaya protease A) were obtained by chromatographically purifying crude papain, from Calbiochem-Novabiochem, which had a mixture of all papaya proteases. Chromatographic separation of the proteases was carried out according to the Dubois method [6]. Actinidin was isolated from Kiwi fruit and purified according to McDowall's method [16]. On SDS-PAGE, all these proteases appeared to have a molecular weight close to 23 000. It was necessary to inhibit these proteases with iodoacetic acid before exposing them to mer-

captoethanol and SDS. Otherwise they autolyse in a few minutes. On native polyacrylamide gel at pH 4.6 in acetate–β alanine buffer the proteases migrated according to their isoelectric points.

Proteases of the papain family have a similar total number of amino acids and considerable amino acid sequence similarity [7,10,11,13,18]. However, their isoelectric points range from 3 to 11 (Table 2). Isoelectric points listed in the Table 2 (except for chymopapain and protease Ω) were taken from Ref. [2]. The complete amino acid sequence of ficin appears to have not been determined. Bromelain and ficinaze glycoproteins [8,9].

2.2. Sulfonate ligands

The ligands used in this study are listed in Table 1. Their structures are shown in Fig. 3. Structures of ligands synthesized in our laboratory were confirmed by proton NMR spectroscopy. The ligands were purified by recrystallization from ethanol–water as sodium salts. All other ligands were purchased from Aldrich Chem. Most were purified by recrystallization.

2.3. Protection from inactivation by acid

Enzymatic activities retained after keeping an enzyme in acid with a protecting ligand, were compared to their activities where protecting ligands were omitted (or added at the end of acidic stress). When the presence of a sulfonate ligand during acid stress preserved enzymatic activity, the ligand was considered to be protective. Ligands enabling the enzyme to withstand acid were assigned a protecting index P (defined below). Some ligands were able to fully protect against significant acidic stress retaining 100% original enzymatic activity.

In most experiments, enzyme concentrations were 1 mg/ml (about 0.04 mM), in 30 mM buffer. Acidic pH stress was imposed by 30 mM formic acid–sodium formate buffer in the pH 2.5–3.5 range, 30 mM hydrochloric acid with sodium chloride in the pH 1.5–2.5 range.

Table 1
Abbreviations of sulfonate ligand names used in this study. The chemical structures and the protection indices P are shown in Fig. 3

Abbreviation	Common names of commercially available ligands
AA	Acid Alizarin Red B, Mordant Red 9
AN	1-Anilino-8-naphthalenesulfonate
AR	Acid Red 97
BB	Brilliant Blue R, Acid Blue 83
BC	Brilliant Crocein MOO, Acid Red 73
BR	Bordeaux R, Acid Red 17
BS	Biebrich Scarlet, Acid Red 66
CG	Crocein Orange G, Acid Orange 12
ND	2-Naphthol-3,6-disulfonate
OG	Orange G, Acid Orange 10
OO	Orange I, Acid Orange 20
OR	Orange ROF, Acid Orange 8
OT	Orange II, Acid Orange 7
PC	Palatine Chrome Black 6BN
PG	Ponceau G
RO	Roccellin, Acid Red 88
SC	Sulfone Cyanin 5R, Acid Blue 11
Abbreviation	**Names of ligands synthesized in our laboratory**
AO	Autumn Orange
AS	Arkansas Scarlet
GM	Gopher Maroon
JO	Jenelle's Orange
JR	Jurga's Red
KR	Kari Red
LE	Lin Red
LR	Little Rock Orange
NO	Nankai Orange
ON	Joel Orange
TO	Tim's Orange
TR	Tim's Special Red

Higher concentrations of hydrochloric acid were used to reach pH below 1.5.

Numbers of sulfonate ligand anions added per enzyme molecule are denoted y_{Ligand}. Optimal protection occurred when y_{Ligand} was equal or a little above the ratio of one sulfonate anion added per positive charge on the protein molecule (Fig. 4). The enzyme was kept in the presence of a ligand and acid for one hour, then neutralized with sodium acetate (usually 0.2 M), or with sodium phosphate buffers. Approximately 100 mg/ml of Dowex 1 anion exchange resin (Aldrich Chem) was added next to remove the sulfonate ligand. Removal of ligand was monitored visually; most of these ligands are

brightly colored. Proteolytic activities were measured after ligand removal. The anionic exchange resin does not bind these proteins. Assay pHs were kept below 7.5 because sulfhydryl groups are susceptible to oxidation in alkaline pH.

2.4. Measurement of enzymatic activity of the sulfhydryl proteases

The proteolytic activity of papain, protease omega, ficin, chymopapain, and bromelain was measured according to Kunitz's method [17] using Hammersten casein as a substrate. The concentration of casein was 10 mg/ml dissolved in 50 mM phosphate buffer of pH 7.0. L-Cysteine (5 mM) was added to protect the sulfhydryl group of the active site from oxidation. After incubation for 20 min at 37°C, the proteolysis reaction was stopped by addition of an equal volume of 1 M perchloric acid. Unhydrolysed casein was removed by centrifugation. The increase in absorbance at 280 nm was proportional to the concentration of amino acids released from casein. Activities of actinidin were measured using hemoglobin as the substrate dissolved in 50 mM formate buffer at pH 3.5. Bromelain activity was also measured using the synthetic substrate *N*-α-CBZ-L-lysine *p*-nitrophenyl ester [21].

2.5. Calculation of the protection index

Fig. 2 shows the remaining proteolytic activities of bromelain after incubation for 1 h in various acidic pHs at room temperature without protecting ligands, and with Bordeaux R ligand (BR). Names of ligands and their abbreviations are listed in Table 1. Their structures are shown in Fig. 3. Relative concentrations of added Bordeaux R protective ligand (y_{BR}) were equal to 100 ligand molecules per bromelain molecule ($y_{BR} = 100$). The Bordeaux R ligand preserved various portions of original bromelain activity when incubated at various pHs. The protection index P compares the capacities of such ligands to protect bromelain enzyme.

An example of determination of the protection index P for Bordeaux R ligand follows (Fig. 2). The area between the plots representing recovered activities with and without the protective ligand is proportional to P. The overall area was calculated by summing areas every 0.5 pH unit. The small areas were obtained by multiplying differences in their ordinate axes between the two plots, by their respective differences in acid concentrations at 0.5 pH change intervals.

For example, at pH 2.5 only 10% of original bromelain activity was recovered without use of the protective ligand. However, 97% of original activity was recovered upon use of the ligand. The difference in the fraction of recovered activity between the results with and without the protective ligand equals $0.97 - 0.10 = 0.87$. This difference is multiplied by the acid concentration multipliers (a.c.m.) derived from the pH scale. The concentration of acid at pH 2.0 is 3.16 times larger than at pH 2.5, and so on. At pH 3.0 there was practically no damage to the enzyme. Hence pH 3.0 is an arbitrary point from which to start calculating the area between two such plots. The numbers (a.c.m.) on the horizontal arrows in Fig. 2 show the differences in acid concentration relative to pH 3.0. These a.c.m. numbers equal 3.16 at pH 2.5, 6.84 at pH 2.0, 21.6 at pH 1.5, 68.4 at pH 1.0, 216 at pH 0.5, 684 at pH 0.0.

The difference 0.87 was multiplied by the first number 3.16. Other fractions of recovered activities at each pH were multiplied by the a.c.m. numbers. The multiplied products were added. The obtained sum was the protection index P. For example, calculation of the protection index P for Bordeaux Red:

$$P = 0.87 \times 3.16 + 0.82 \times 6.84 + 0.74 \times 21.6 + 0.43 \times 68.4 + 0.0 \times 216 + 0.0 \times 684 = 53.8.$$

Ligands producing protection indices smaller than 10 are considered weak protectors. Ligands

yielding P between 10 and 100 are intermediate, and ligands with P above 100 are strong protectors. Ligand protection indices (P) are listed next to their chemical structures in Fig. 3.

2.6. Isothermal titration calorimetry

Binding of several aromatic sulfonates to polyamino acids was measured by titration calorimetry as described previously [14]. Concentrations of cationic amino acid in the cell of titration calorimeter (Microcal Omega) were 1 or 3 mM. Concentrations of sulfonate ligands were 10 or 30 mM in the 250 μl injection syringe. The pH in both the cell and the syringe were adjusted to 2.0 by adding 10 mM HCl, and 20 mM NaCl. Heats of dilution of these organic sulfonates with bulky nonpolar groups were substantial (up to 3 kcal/mol) and were subtracted from the overall measured heats to yield the enthalpies of ligand binding.

2.7. Protection of catalase by little rock orange ligand

Catalase enzyme was purchased from Sigma. For protection assays the catalase enzyme concentration was 2 mg/ml in 30 mM formate–HCl buffer, pH 2.5–3.0. Little Rock Orange ligand was added at various y_{LR} levels before addition of acid, or at the end of acidic stress. After the stress, the ligand was removed with Dowex 1 anion exchange resin. The remaining activity was measured using hydrogen peroxide substrate.

2.8. Protection from temperature inactivation

Bromelain enzyme solutions (at pH 1.7 in HCl, and pH 4.8 in acetate buffer) were incubated in a 65°C water bath for various intervals (1 to 60 min), then rapidly cooled to room temperature. Proteolytic activities were measured as described above.

3. Results

3.1. Protection of papain family enzymes from inactivation by acid

Enzymes of the papain family are stable for a few hours in pH ranges from 4 to 8. Above pH 8 the cysteine sulfhydryl group of their active sites oxidize, inactivating these enzymes. They rapidly lose activity in the pH range below 4 from acid denaturation. At pH 1.8, protease omega loses 50% of its activity in 40 s. Bromelain loses 50% of its activity in 2 min. The rate of loss of the secondary structure of protease omega at pH 1.8, monitored by circular dichroism, was much slower: 50% decrease in ellipticity at 222 nm occurred in 15 min. Similar behavior was observed earlier for many proteins by other researchers. For example, the inactivation rate of papain was found to be 100-fold higher than the unfolding rate [25]. Therefore monitoring enzymatic activity appears considerably more sensitive to demonstrate protection, than monitoring loss of enzyme secondary structure by circular dichroism.

Addition of sulfonate ligands to the bromelain solution before subjecting it to acidic conditions often leads to the protection of this enzyme, yielding nearly 100% recovery of enzymatic activity after acid neutralization followed by ligand removal. For example, Jurga's Red ligand (structure is shown in Fig. 3) protects papaya protease omega from pH 1.7 acid (Fig. 1a). Without added protective ligand, protease omega was irreversibly inactivated at pH 1.7 in a few minutes. Addition of a weakly protective ligand like Orange II only negligibly slows inactivation of the enzyme.

3.2. Protection from inactivation by elevated temperatures

Temperatures above 50°C damage bromelain. At 65°C, pH 4.8, the enzyme is irreversibly inactivated in one hour (Fig. 1b). Addition of Jurga's Red ligand before the stress cycle retained most of enzyme's activity for an hour.

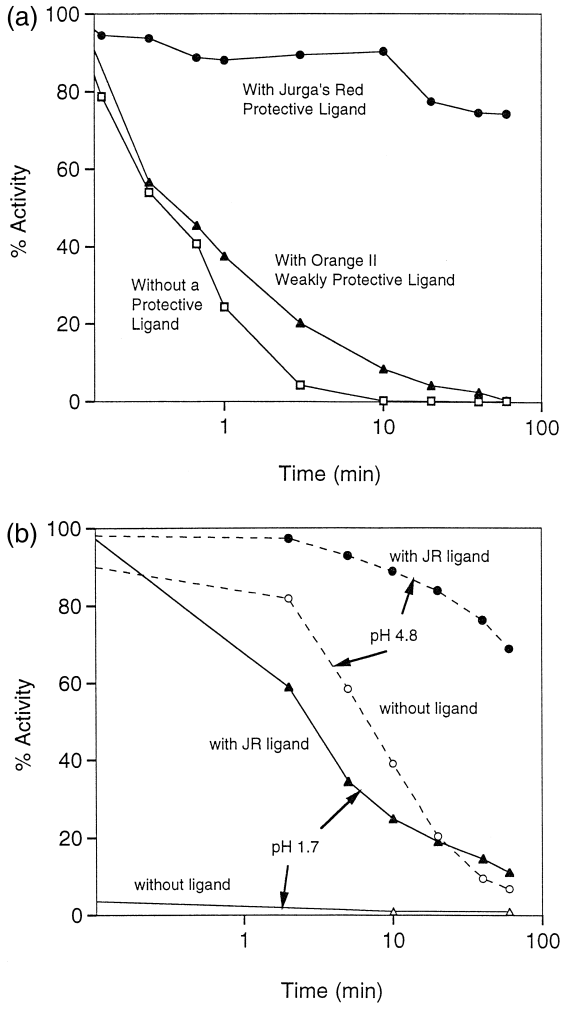


Fig. 1. (a) Protection of papaya protease omega against acidic pH 1.7, 37°C by strongly protecting ligand Jurga's Red (●), weakly protecting ligand Orange II (▲), and without a ligand (□). (b) Protection of bromelain by Jurga's Red ligand from temperature-inactivation of 65°C at pH 4.8 (dashed lines) and at pH 1.7 (solid lines). At both pHs more activity is preserved with added protecting ligand (▲—pH 1.7, ●—pH 4.8) than without added ligand (△—pH 1.7, ○—pH 4.8).

Even at pH 1.7, Jurga's Red ligand preserved part of bromelain's activity from 65°C stress.

Various ligand abilities to protect bromelain from elevated temperatures paralleled their abilities to protect from acid at 37°C. Weak protectors from acid-inactivation were also weak protectors against temperature-inactivation (e.g. Orange II). There were exceptions, however, where protectors from acid-inactivation had no protecting ability for temperature inactivation as in case of Little Rock Orange.

3.3. Relation of ligand structure with protecting ability

Protection indices (P) were experimentally determined for each of the ligands. Fig. 2 plots the retained activities of bromelain after incubating it 1 h in buffers of various pH. At pHs below 4, the enzyme becomes inactivated by acid. Bordeaux R ligand preserves most of bromelain's activity at pH 2 and even lower. The area between the two plots shown in the Fig. 2 is proportional to the protection index P. Increases in the P index indicate enhanced ability of such ligand to protect the enzyme from acid inactivation.

Table 1 lists names of the 29 selected ligands, and Fig. 3 shows their structures. Protection indices, P, are listed next to the abbreviated names and structures of each ligand in Fig. 3. Ligands of related structures with respect to

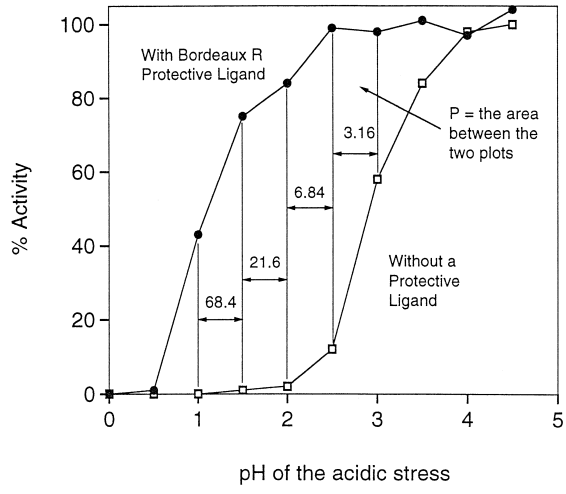


Fig. 2. Determination of the protection index P. Without a protective ligand bromelain is inactivated when pH is below 3 (□). With Bordeaux R protective ligand bromelain withstands about 54 times more concentrated acid and is inactivated at pH below 1 (●). The calculated P is equal to the area between the two plots which is equal to 54.

their substituents on the azoaromatic groups are arranged vertically in order of decreasing protective index P. Structural differences between closely related, homologous structures are shown in bold print. These differences appear to be significant factors determining ligand ability to protect enzymes.

The first column of Fig. 3 shows structures closely related to Orange II ligand (OT). Orange II itself is a weak protector of bromelain ($P = 1.7$). Addition of various aliphatic groups to the naphthalene ring in the sixth position dramatically alters the ability of this ligand to protect bromelain. Addition of the tertiary butyl group increases the protection index by about 50 times (Little Rock Orange has P of 73). Addition of ethyl or propyl groups increased the protection index of related ligands. On the other hand, hexyl or propionate substituents had no notable effect on protective ability by the Orange II parent ligand. Minor changes in ligand structure thus engendered dramatic changes in protective abilities of the Orange II homologues from acid-inactivation.

The second column of Fig. 3 compares another group of monosulfonate azo dyes, which are also closely related to Orange II. Addition of a conjugated benzene ring to Orange II increased its ability to protect bromelain (Rocellin's $P = 81$). Strikingly, addition of just one methyl group, forming Orange ROF's (OR, $P = 27$) ligand structure, strongly increased Orange II's ability to protect bromelain. The ring position of the added methyl group appears to be important: Tim's Special Red (TR), the structural isomer of OR, does not protect bromelain. The additional hydroxyl group added to Roccellin (RO) decreases its protection index resulting in no protection by Palatine Chrome Black 6BN (PC, $P = 2.9$). Changing the position of the hydroxyl group of Orange II (resulting in Orange I (OO)) or the position of sulfonate group (resulting in Crocein Orange G (CG)) had no effect on protection.

The third column compares the protective abilities of some disulfonates. 2-Naphthol-3,6-disulfonate (ND)—a ligand with a small hydrophobic group—has no protecting ability. Addition of increasingly large aliphatic or aromatic groups increases the ability of related sulfonates to protect bromelain. For example, Gopher Maroon (GM, $P = 110$) has its phenyl group attached to Joel Orange (ON, $P = 5$), and is the strongest protector in the third column. It is the largest of the ligands listed in the third column.

The fourth column shows structures of the strongest protectors known to us—Jurga's Red, and Acid Red 97. Jurga's Red is the only ligand enabling bromelain to withstand 1 M hydrochloric acid for 1 h at 37°C with recovery of 50% or more original activity. Jurga's Red ligand was the most efficient synthetic protective ligand for the papain family enzymes in general. Orange G (OG) has no protecting ability ($P = 0.9$). Addition of the azobenzene group to OG (resulting in Brilliant Crocein MOO [BC, $P = 14$]) increases OG's ability to protect bromelain, but only by a factor of about 3. However, addition of two methyl groups to OG (resulting in Ponceau G (PG, $P = 44$)) increases protection by a factor of about 9. Furthermore, the Biebrich Scarlet ligand (BS, $P = 110$) has its sulfonate groups located in different positions (compared to BC) and is a very strong protector.

A few more structures are shown in column 5 of Fig. 3. Coomassie Brilliant Blue R (BB) has no protecting ability. ANS (AN) is also a poor protector, but Sulfone Cyanin 5R which is similar to ANS dimer, has profound protecting ability, similar to Kari's Red, shown on the top of column 5.

Clear reasons for the complicated behavior of these closely related ligands are not precisely known. The results are rather empirical, not predictable from first principles. However, some trends in ligand structures can be observed based on results seen so far.

1. The hydrophobic tail of ligands of this kind need to be large. Ligands with larger hydrophobic groups generally are stronger protectors.

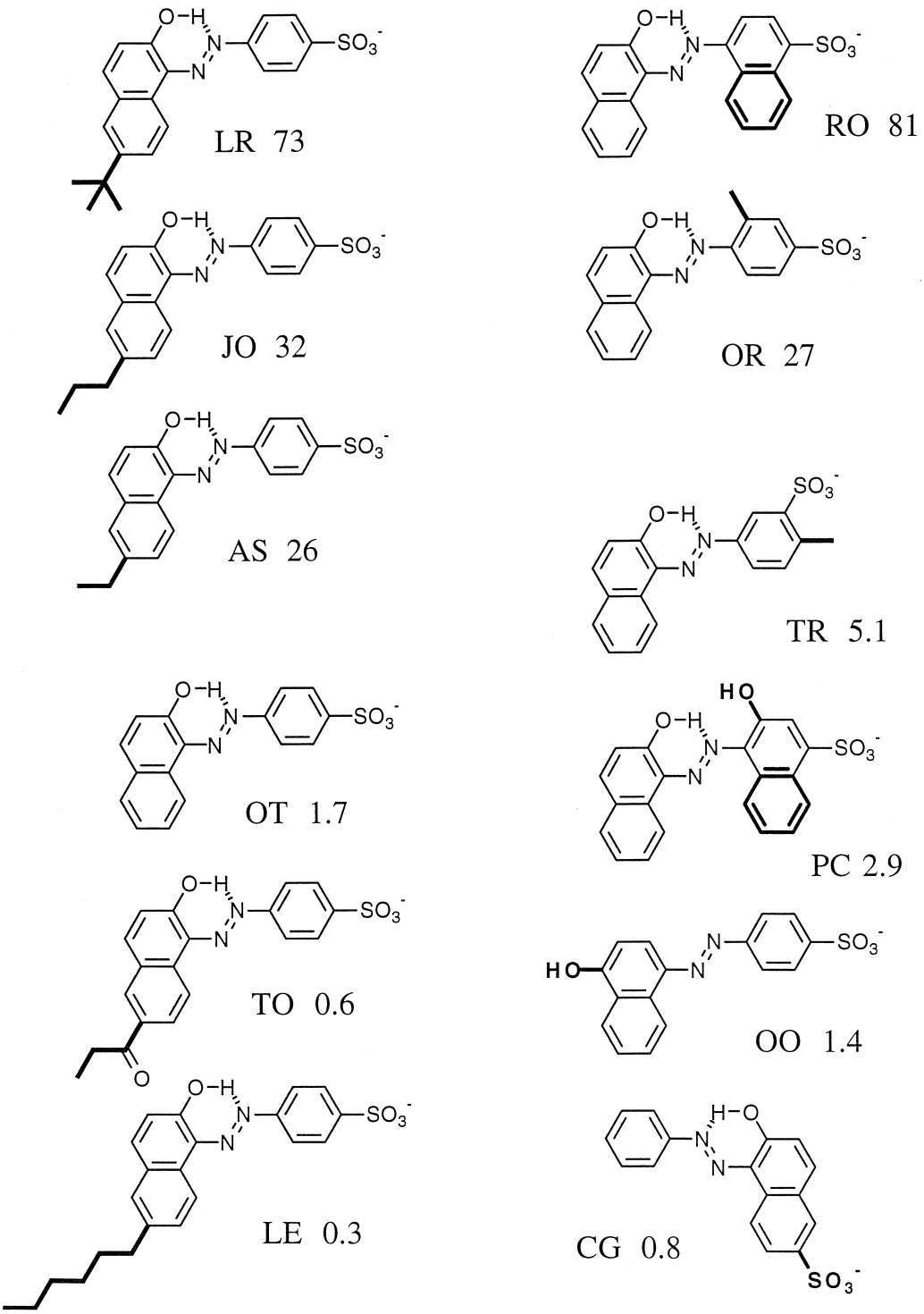


Fig. 3. Structures of the azoaromatic sulfonate ligands, two-letter abbreviations of the names, and the protection indices *P* for bromelain. Similarly structured ligands are arranged in columns with stronger protectors above the weaker ones. Notable salient differences of adjacent structures are shown in bold. The structure of the best protecting ligand—Jurga's Red (JR)—is shown at the top of the fourth column.

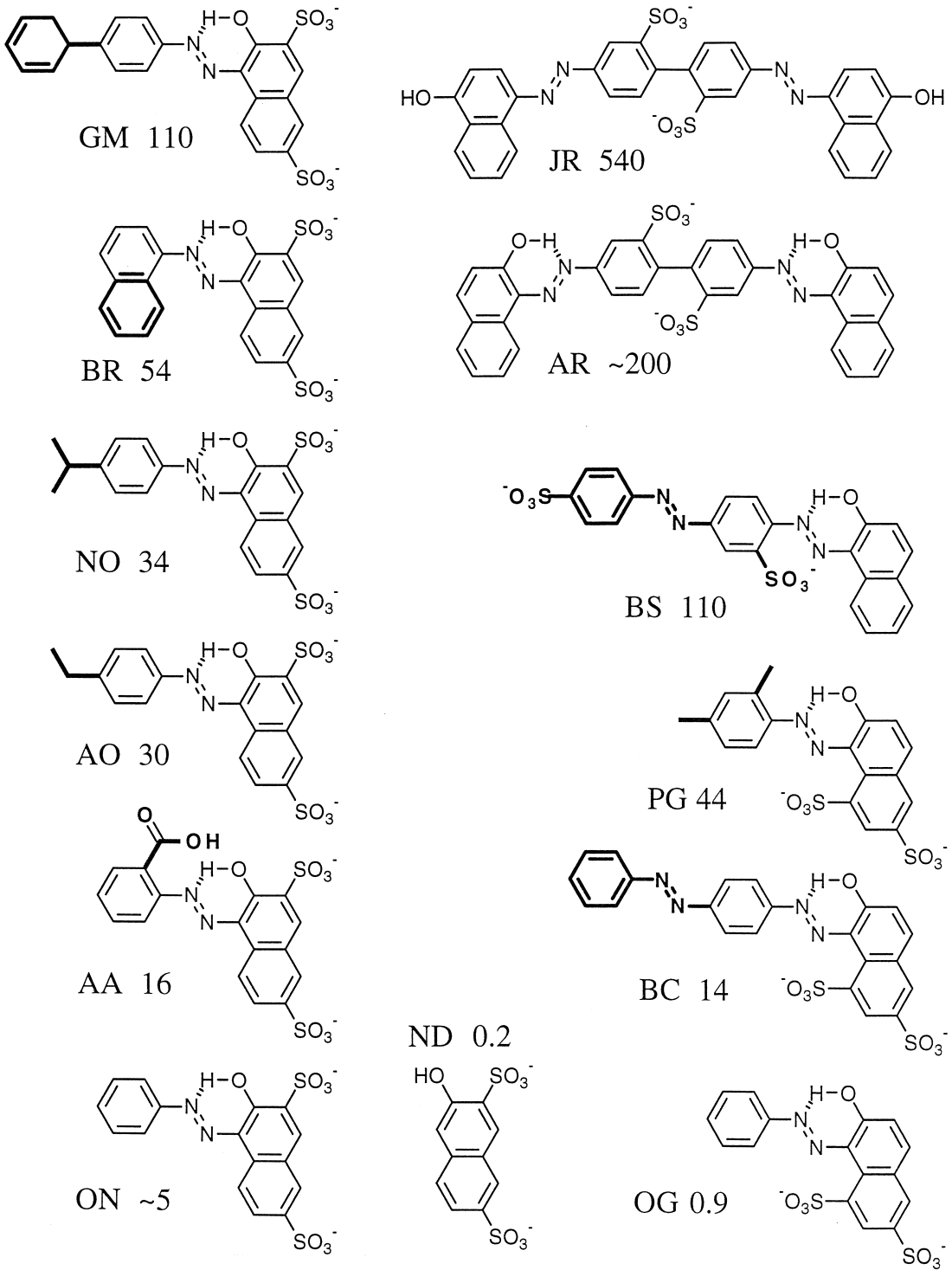
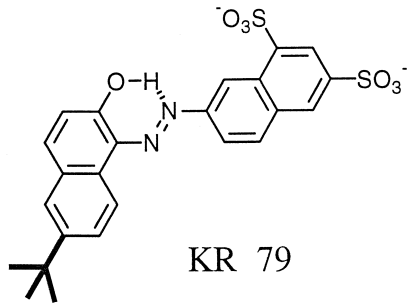
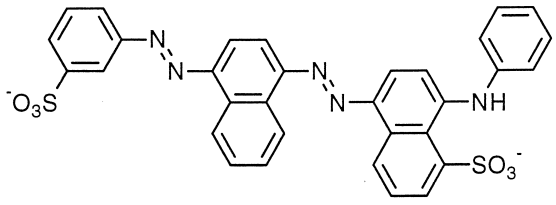


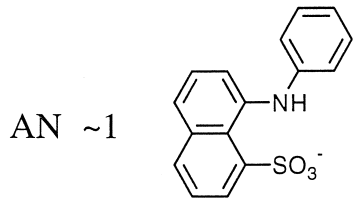
Fig. 3 (continued).



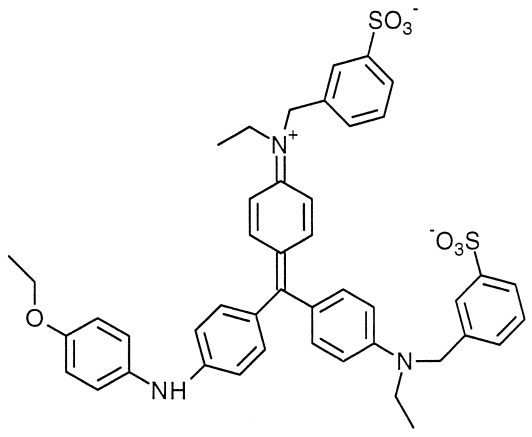
KR 79



SC 82



AN ~1



BB 1.4

Fig. 3 (continued).

2. A hydrophobic tail which is too bulky (as in the case of Lin Red), possibly interferes with the binding due to steric hindrance, therefore reduces the protective power of such ligands.

3. Even one small methyl group substituent may impact protection (as in the Orange ROF case).
4. Disulfonates are generally stronger protectors than monosulfonates.
5. Disulfonates with two tails, such as Jurga's Red, are generally stronger protectors than those with one organic tail (Gopher Maroon).

The same protection experiments were partly carried out using other sulfhydryl proteases. Protection indices for many ligands differed for each protease. However, the general trends were quite similar. Jurga's Red ligand was strongest protector of ficin, protease omega, papain, and chymopapain. In contrast the Little Rock Orange ligand was not as protective for these proteases as in case of bromelain.

3.4. Protection by non-aromatic sulfonate and sulfate ligands

Various aliphatic and carbohydrate sulfonate and sulfate ligands were tested for their protective abilities for papain family proteases.

Synthetic aliphatic ligands, such as dodecyl sulfate, tetradecyl sulfate, octadecyl sulfate, hexadecane sulfonate, offer little protection for bromelain.

However, several naturally occuring ligands, such as sucrose octasulfate, crude heparin (sulfated poly carbohydrate) exhibit mild protection ability with a protection index P of approximately 10 to 30 for bromelain. Sucrose without sulfate substituents did not protect. Such behavior indicates that sulfated carbohydrates may play protective roles towards proteins in vivo.

3.5. Dependence of protection on relative ligand concentrations

Protection of proteases by azoaromatic sulfonate ligands depends on the amount of ligands bound, which in turn depend on the amount of ligand added. Optimal levels of relative ligand concentrations must be reached for maximum protection. The ratio of molar added ligand and enzyme concentrations is denoted y_{Ligand}. Fig. 4 shows that variable amounts of Jurga's Red ligand protect actinidin, papain, bromelain, and protease omega enzymes. Jurga's Red ligand was not a strong protector against acid inactivation (pH 1.7) for actinidin (only up to 10% activity was recovered). About 25% of papain activity was recovered. About 60% of protease omega original activity was recovered. However, bromelain was best protected by Jurga's Red yielding nearly 100% of original activity. It appears that levels of protection depend both on the individual structures of protein and ligand molecules.

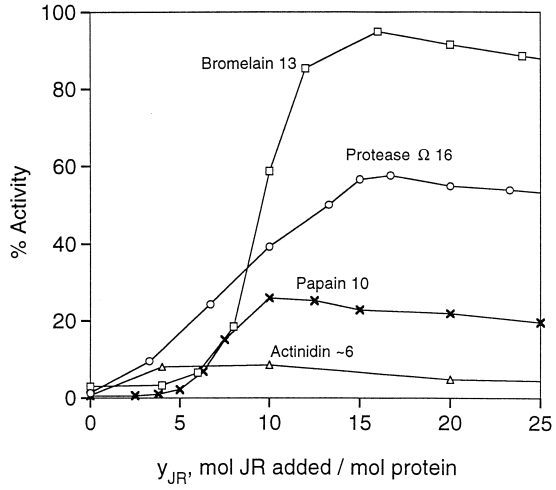


Fig. 4. Dependence of protection on the relative concentration of added Jurga's Red ligand for the sulfhydryl proteases: bromelain (□), papaya protease omega (○), papain (×), and actinidin (△).

From Fig. 4 we can determine the least number of Jurga's Red ligand molecules to add per enzyme molecule (y_{JR}) necessary to achieve maximum protection. These numbers are shown in Fig. 4 and have the approximate values: 6 for actinidin; 10, papain; 13, bromelain; and 16, protease omega. Further addition of this protecting ligand does not increase the level of protection. Jurga's Red ligand molecule has two sulfonate anionic groups. The y values of maximum protection correlate with the numbers of positive charges per protein molecule (shown in bold in Table 2). Protease omega has 38 positive charges and binds 16 Jurga's Red ligand

Table 2
Characteristics of sulfhydryl proteases, numbers of arginine residues (Arg), histidines (His), lysines (Lys), total number of the positive charges, aspartic acids (Asp), glutamic acids (Glu), total number of the negative charges, total number of charges per protein molecule, the total number of amino acids (a.a.), isoelectric points (pI), and Brookhaven protein data bank (PDB) codes of solved protein structures

Characteristic	Actinidin	Bromelain	Papain	Chymopapain	Protease Ω
Arg content	5	6	12	5	11
His content	1	1	2	3	4
Lys content	6	15	10	21	22
Total number of positive charges	**13**	**23**	**25**	**30**	**38**
Asp content	16	8	6	6	3
Glu content	11	9	7	7	11
Total number of negative charges	28	18	14	14	15
Total charges	41	41	39	44	53
Total number of amino acids	220	212	212	218	216
pI	3.1	9.6	8.8	~ 10	~ 11
PDB code	2ACT	–	9PAP	1YAL	1PPO
y_{JR} necessary for maximum protection	~ 6	13	10	–	16
y_{JR} anionic group necessary for maximum protection	**~ 12**	**26**	**20**	–	**32**

molecules, bearing a total of 32 negative charges. Bromelain has 23 positive charges and binds 13 disulfonate molecules, equivalent to 26 negative charges. Papain has 25 positive charges, but maximal protection is observed when 20 anions are bound from 10 Jurga's Red dianions. Actinidin has 13 positively charged groups and maximal protection is achieved by 12 bound anionic groups (Table 2). Correlations between the number of positive charges on each protein molecule and the minimal number of organic sulfonate anions, necessary to achieve maximum protection, is indicative of ion pairing. The discrepancies here may be due to errors in estimating protein concentrations. Impurities present in crude bromelain may also bind sulfonate molecules.

3.6. Thermodynamics of sulfonate ligand binding to proteases and cationic polyamino acids

In order to further confirm the binding mechanism as ion pair formation between sulfonates and cation groups on proteins, binding of various aromatic sulfonate ligands to proteins and

Table 3
Molecular weights of the organic moieties (not including MW of the sulfonate group) of the ligands (MW), protection indices for bromelain (P), stoichiometries of binding per positively charged amino acid (n), association constants (K_b), and the enthalpies (ΔH) of various aromatic sulfonate binding to cationic polyamino acids and BSA as determined by titration calorimetry at pH 2.0, 25°C

Aromatic sulfonate ligand	MW	P	Polyamino acid or protein	n (mol/mol)	K_b (M^{-1})	ΔH (kcal/mol)	ΔS (cal/(mol · deg))
Benzene sulfonate	77	1	poly-Arg	~ 1	~ 10	~ −0.7	~ 2
Naphthalene-1-sulfonate	127	1	poly-Arg	~ 1	~ 100	~ −0.5	~ 7
p-Azobenzene sulfonate	181	1	poly-Arg	0.9	20,000	−4.5	4.7
ANS[a]	218	1	poly-Arg	0.88	250,000	−6.0	4.7
Orange II	247	1.7	poly-Arg	0.78	300,000	−8.6	−3.7
Arkansas Scarlet	275	26	poly-Arg	0.90	2,000,000	−6.5	7.0
Little Rock Orange	303	73	poly-Arg	0.88	3,000,000	−5.8	10
Orange II	247	1.7	poly-Lys	1.03	100,000	−5.7	3.7
Naphthalene-1-sulfonate	127	1	BSA	~ 110[b]	–	−0.6	–
Arkansas Scarlet	275	26	BSA	77[b]	–	−4.1	–

[a] These parameters of ANS binding, and also calorimetry of ANS binding to sulfhydryl proteases, were described in greater detail earlier [14].
[b] Stoichiometry per molecule of BSA which has 100 positively charged amino acids: 24 Arg, 17 His, 58 Lys, and 1 N terminal charge.

cationic polyamino acids was measured by isothermal titration calorimetry. Calorimetric titration curves were similar to the curves of ANS binding to cationic polyamino acids [14]. Thermodynamic parameters for several sulfonate ligand binding to polyarginine, polylysine, and bovine serum albumin are shown in Table 3. Ligands are listed in the order of increasing molecular weight of the organic group of each ligand not including molecular weight of the sulfonate head.

The stoichiometry of binding was close to one anionic ligand bound per positively charged amino acid in all cases. Association constants increased as the molecular weights of the organic moiety increased. Enthalpies of binding generally were more exothermic for ligands of larger molecular weights. Entropies of binding were calculated from the association constants and the enthalpies of binding. The entropies usually were positive contributing favorably to the binding reaction. The thermodynamic constants of binding of these sulfonate ligands to polyarginine did not change in the pH region from 2 to at least 8 (where there is no change in ionization of the guanidinium group), indicating that ion pairs form in the whole pH region where arginine is positively charged.

3.7. Protection is closely related but not limited to precipitation

When optimum amounts of anionic ligands are added to a positively charged protein, protein–ligand complexes coprecipitate. Proteins become protected while in their precipitated, water-insoluble form. Fig. 5 shows a correlation of ficin protection with its precipitation. At pH 2.2, damaging conditions to the unprotected enzyme, only a small part of activity is recovered in the supernate. When a sufficient amount of Jurga's Red ligand is added, its complex with ficin coprecipitates, and over 80% of ficin's activity is recovered. At pH 4.2, non-damaging conditions, most of enzyme's activity remains in the supernate until the ligand is added. Up to

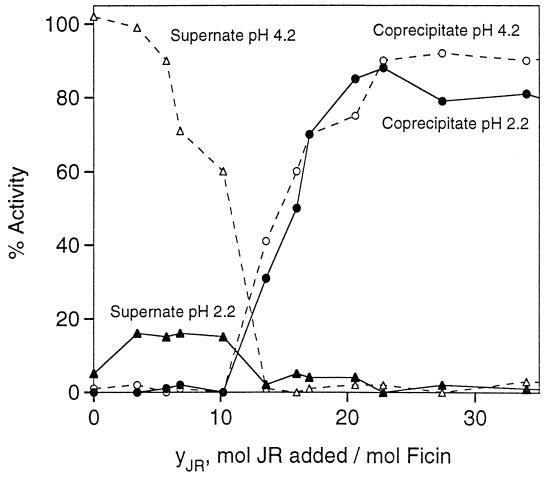


Fig. 5. Relation of protection to coprecipitation of the ficin enzyme by Jurga's Red ligand. At non-damaging pH 4.2 (dashed lines) ficin activity is found mostly in the supernate (△) at y_{JR} below about 12, and in the coprecipitate (○) at y_{JR} above about 12. At damaging pH 2.2 (solid lines) ficin activity is mostly lost in the supernate (▲) at y_{JR} below about 12, but found in the coprecipitate (●) at y_{JR} above about 12.

100% of its original activity is recovered from the coprecipitate.

Coprecipitation occurs at similar y values both for pH 2.2 and 4.2. It indicates that in this pH region the composition of the protein–ligand complex remains constant (e.g., 20 sulfonate groups per enzyme molecule). The stability of the complex is part of the origin of protection. Only the more strongly binding ligands maintain constant composition of the complex, which in turn is a more strongly protected complex.

Almost all of the 29 ligands shown in Fig. 3 (except 2-naphthol-3,6-disulfonate) coprecipitated the papain family proteases. The 2-naphthol-3,6-disulfonate has a relatively small hydrophobic tail, apparently inadequate to coprecipitate and protect. Only about half of the ligands shown in Fig. 3 protected bromelain. Thus precipitation is not completely sufficient to aquire protection.

3.8. Dependence of protein–ligand complex formation on pH

The composition of the protein–ligand complex depends on pH of the medium. Numbers of

bound ligands per protein molecule correlate with the overall cationic charge of the protein molecule (Fig. 6), hence increases as pH decreases. For example, papain molecules have about 9 ANS anions bound in the coprecipitate at pH 7 and 14 at pH 4.7. The charge of the papain molecule at these pHs is approximately +6 (calculated from its amino acid composition) and +12, respectively. The number of bound sulfonate anions is a little larger than the formal charge. Such occurs upon ligand binding, because proteins tend to bind some protons from water molecules to neutralize the excessive negative charge, resulting in increased pH. This phenomenon is the Scatchard–Black effect [19].

A similar trend is observed with actinidin which has a very low isoelectric point of 3.1. Due to the Scatchard–Black effect, actinidin binds about six ANS anions at pH 4 even though its normal charge without ligands is about −3 at pH 4. ANS was selected to illustrate this concept because it is generally considered to bind to hydrophobic groups on the surface or inside of the protein molecule judged by an increased yield of ANS fluorescence. Experiments with protease coprecipitation confirmed the earlier conclusion that ANS primarily binds to proteins by forming an ion pair [14].

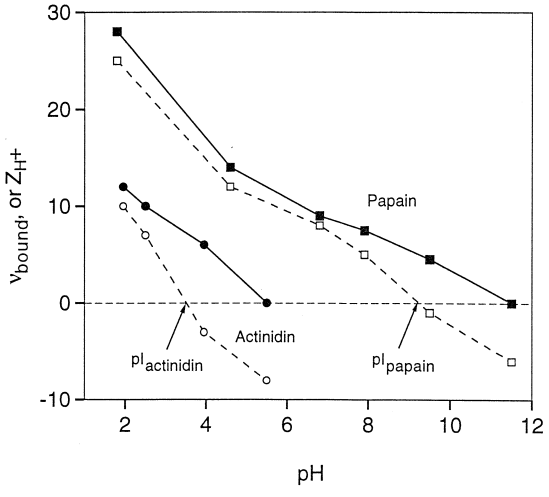


Fig. 6. Actinidin and papain pH titration curves (dashed lines). The isoelectric points (pI) are about 3.5 and 9, respectively. Dependences of the number of 1-anilino-8-naphthalene sulfonate (ANS, AN) anions bound per actinidin (●) and papain (■) molecules on pH are shown as solid line. These numbers of bound ANS correlate with the positive charges of the protein molecules (actinidin—○, papain—□).

Interestingly ANS is a ligand of intermediate binding strength, unable to protect any of these proteases from acidic stress. Such proteases form complexes of variable ligand–protein composition depending on pH. Strongly binding ligands like Jurga's Red tend to bring the coprecipitate's composition to a relatively constant value over a considerable pH range. Formation of strong complexes, yielding compositions independent of the starting pH, appears to be part of the reason why Jurga's Red is such a strongly protecting ligand.

3.9. Protection of catalase by little rock orange ligand

Catalase is a much larger enzyme (MW 250,000) than the proteases described above. It is a subunit enzyme, a tetramer, dissociating at pH < 3.0, with complete loss of activity [22].

Unprotected catalase loses 50% of its activity in about 30 s at pH 2.50 (25°C), in about 6 min at pH 2.75, and in 2 h at pH 3.00. Catalase contains 51 cationic amino acids to contribute to overall cationic charge in the acid pH regions. When the amount of added Little Rock Orange (y_{LR}, moles LR/mol catalase) was lower than 50, catalase lost activity during acid stress. However, when y_{LR} exceeded 50, dramatic increases in the half-inactivation times were observed. At $y_{LR} = 147$ about 24 min were required to inactivate catalase to half its original activity at pH 2.50 ($24/0.5 = 48$ fold), and about 85 min at pH 2.75 ($85/6 = 15$ fold). Little Rock Orange prolonged catalase's life by a factor of 48 times at pH 2.50 and by a factor of 15 times at pH 2.75. Thus catalase, a large tetrameric protein, is protected by aromatic sulfonate ligands from acidic inactivation.

4. Discussion

Azoaromatic sulfonate ligands protect sulfhydryl proteases from inactivation by acid and elevated temperatures. For example, ligand-protected bromelain was incubated in 1 M HCl for 1 h, and most of its activity was recovered afterwards. Unprotected bromelain is nearly instantly destroyed in strong acid. Protection was not observed while the protein was in solution, and coprecipitation appears required for protection. However, simply coprecipitation alone is not sufficient for protection. Indeed, precipitation of proteins is a common criterion for denaturation. Of about 50 ligands that were studied here, only about 15 protected bromelain. Protective capacity of ligands depends on ligand structure: ligands with larger hydrophobic groups are better protectors than those with smaller hydrophobic groups, disulfonates are better protectors than monosulfonates.

Protection of six sulfhydryl proteases with similar tertiary structures was compared. Monosulfonate ligands, such as Little Rock Orange, were not protective for actinidin or protease omega. These two proteases have the lowest and highest surface positive charge densities respectively of the six proteases. Bromelain and papain, bearing intermediate surface charge densities were most protected against acid stress. Actinidin was not protected, likely because actinidin has insufficient cationic groups which are required for ion pair formation. Protease omega was protected but little, possibly because it has too many positively charged groups for optimal ligand binding. Excessive numbers of

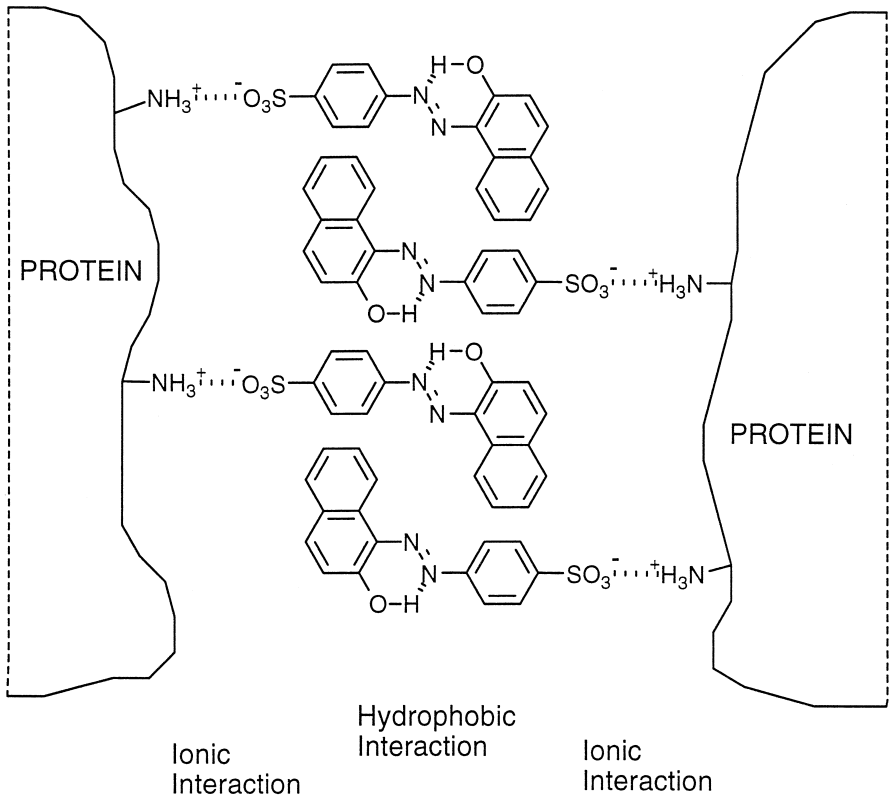


Fig. 7. Matrix coprecipitate formation. Anionic sulfonate ligands are bound to the positively charged groups on a protein, forming salt bridges (ion pairs). Hydrophobic tails of sulfonate ligands are bound primarily to each other by hydrophobic interactions. Protein–protein interactions (not shown) are also possible.

ligands cannot be accommodated, disrupting the structure of protease omega. The sulfhydryl proteases are monomeric enzymes. However, catalase, a tetramer susceptible to acid denaturation and dissociation [22], is also protected by at least one of these ligands.

Sulfonate ligands titrate protein cationic groups and reach maximum protection at the end of titration where the numbers of bound ligands per protein molecule (ν_{Ligand}) are close to the overall cationic charge of the protein (Z_{H^+}). Proteins and ligands mutually coprecipitate at the end of titration. This behavior supports the conclusion that ion pairs form between each ligand negative charge and protein positive charges. Our earlier studies showed that 1-anilino-8-naphthalene sulfonate (ANS, AN) bound to the positive charges on a protein molecule by forming ion pairs [14]. Azobenzene sulfonate ligands also form ion pairs with the positively charged groups on each enzyme molecule as seen from the stoichiometry and thermodynamics of ligand binding to cationic polyamino acids. The size of the organic tail is a determinant of ligand affinity towards a protein. Larger organic anions are also the stronger binding ligands (Table 3), stronger protection and coprecipitation agents.

Large organic nonpolar groups of the ligands displace considerable amount of water from immediate environment of ion pairs. Although water is often cited as biocompatible solvent, an excess of water inside and on protein surface may be an aid to denaturation, if it aids conformational expansion of proteins. Large organic anions, especially sulfates and sulfonates, exert protection through: (i) neutralization of positive charge, and (ii) displacement of water and solvent reorganization, to allow acid expended proteins to retreat to a compact, protected form. Nonpolar groups interact with each other drawing protein–ligand complexes together, shown in Fig. 7.

Organic ligand binding is accompanied by large upward shifts in pH, usually called the Scatchard–Black effect. When a protein's positively charged group binds negatively charged sulfonate by an ion pair, the complex acquires one negative charge. To reestablish electroneutrality, a carboxylate group binds a proton from water, releasing hydroxide anion to the solvent, an alkaline shift in pH. Scatchard–Black effects are readily measured by the pH shift and are well studied [20]. These effects add another means for observing formation of ion pairs.

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