Vibrational Studies of the Disulfide Group in Proteins

VII. Normal Mode Analysis of the Raman Spectra of Erabutoxin, γ-II Crystallin and Immunoglobulin

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Normal mode calculations have been made on the known structures of disulfide bridges in erabutoxin b, a form of γ-II crystallin, and fragments (Fab, variable-domain of Bence-Jones proteins) of immunoglobulins. Previously obtained Raman spectra of these molecules are analyzed in terms of general correlations derived from normal mode calculations and also the results of calculations on these particular structures. This results in more specific conclusions about these disulfide bridge structures and their changes than was possible on the basis of previous empirical correlations.

INTRODUCTION

In previous papers we obtained a conformation-dependent scaled ab $initio$ force field for the disulfide bridge[1-3] and applied it to normal mode calculations of SS stretch, $v(SS)$, and CS stretch, $v(CS)$, frequencies in known protein S—S bridges[4] and to the determination of general correlations between such modes and the geometry of this bridge.[5,6] We were able to show that $v(SS)$ and $v(CS)$ depend not only on the dihedral angles of the bridge, viz. $C^\beta SSC^\beta(\chi^3)$, $C^\alpha C^\beta SS(\chi^2)$ and $NC^\alpha C^\beta S(\chi^1)$, but also on the torsion angles of the adjacent peptide groups, viz., $CNC^\alpha C$ (ϕ) and $NC^\alpha CN$ (ψ). Such an approach extends the useful but rough information that can be obtained from simple model compound correlations,[7,8] and represents the deepest understanding that we can achieve in relating vibrational spectra to the structure of the disulfide bridge.

In this paper, we apply this approach to a discussion of disulfide bridge structure in several proteins. Our aim is to examine how such computational correlations[5,6] as well as normal mode calculations on known structures can provide deeper insights into S—S bridge conformation than were possible from empirical model compound studies.[7,8]

Normal mode calculations were done, as before,[4-6] on a model for the bridge, viz. (CCONH)(CNHCO)CHCH$_2$SSCH$_2$CH(CONHC)(NHCOC), using our ab $initio$ S—S force field[3] together with our empirical force field for the peptide group.[9] With respect to $v(SS)$, we designate general bridge conformations by the $\chi_1{}^2\chi_2{}^2$ values (χ^3 is assumed to be constant at $ca.$ $+90° \equiv$ G), these being 0° (C), 30° (A), 60° (G), 90° (B), 120° (S), 150° (D), 180° (T), and their negative values (D', S', B', G' and A'). With respect to $v(CS)$, we designate the conformation by $\chi^2\chi^1\phi,\psi$; χ^1 is indicated by the atom (N, C or H) $trans$ to S across the $C^\alpha C^\beta$ bond, and ϕ,ψ has in the general case been computed at

values for the α-helix (α: $-57.4°$, $-47.5°$), β-strand (β: $-138.4°$, $135.7°$), and extended helix (ε: $-80°$, $142°$ [6]). For specific structures, of course, all of the x-ray-determined angles were used in the normal mode calculation, these generally being obtained from the Protein Data Bank.[10]

ERABUTOXIN

Raman spectra have been obtained for aqueous solutions of erabutoxin b[11] (Eb), a small protein of 62 amino acid residues and four S—S bridges. A high-resolution x-ray structure is also available for this molecule.[12] Two bands were observed in the $v(SS)$ region, at 511 and 524 cm^{-1} with an intensity ratio of $ca.$ 3:1,[11] and model compound correlations[7,8] led to the conclusion that three of the S—S bridges were in the GG conformation and one was GT. A strong broad band at 657 cm^{-1} was assigned to $v(CS)$ of the S—S bridges (there is no methionine in Eb) and attributed to a $\chi^1 = H$ conformation.[11] No comment was made about weak bands at $ca.$ 620, $ca.$ 700, and $ca.$ 725 cm^{-1} (a medium band at 758 cm^{-1} is due to tryptophan).

Our classification of $v(SS)$, based on normal mode calculations of 92 S—S bridges in 25 known protein structures[5] and also of more general structures,[6] indicates that a band at 511 cm^{-1} should be associated with a conformational class, 1b, of range 506–512 cm^{-1}, in which $\chi_1{}^2$ and $\chi_2{}^2$ have (mostly) the same chirality; the observed structures in this class are, for $\chi^3 = G$, mainly GB, GG, B'S', BB, B'B' and GA (plus some SB' and SS') (this is in contrast to class 1a, in which the chiralities are always opposite, observed in GB', G'B, G'G and B'B, and the range is lower, 503–506 cm^{-1}). The known conformations of three of the S—S bridges in Eb satisfy this condition,[12] and our calculations of their $v(SS)$ at 508, 510 and 510 cm^{-1} (see Table 1) are in

Table 1. Disulfide bridge frequencies (in cm^{-1}) of erabutoxin b

r^a	v_{obs}^b	Cys 3–Cys 24 G'G'G'[c]		Cys 17–Cys 41 G'G'B'		Cys 45–Cys 54 GGT		Cys 55–Cys 60 BGB	
		$C\beta^d$	$C\beta$	C_r	C_ε	$N\beta$	C_ε	$H\beta$	$H\alpha$
ϕ		−128.7	−130.0	−75.0	−86.9	−136.9	−114.3	−146.5	−84.4
ψ		159.2	161.7	172.2	151.6	93.9	150.2	160.0	−13.9
χ^1		−65.2	−60.7	−47.1	−74.1	−174.3	−61.5	53.5	63.1
χ^2		−67.8	−59.8	−54.1	−91.8	66.7	178.5	88.9	88.1
χ^3		−85.4		−82.7		83.6		85.6	
CS	∼750 w(?)					745 (63)[e]			
	∼725 w			720 (37)				723 (28)	
					715 (37)				
		709 (27, 14)[f]							
	∼700 w	707 (27, 14)							
							673 (37)		674 (45)
	657 s							659 (59)	
	620 w								
SS	524 w					527 (75)			
	511 s		508 (97)	510 (96)					510 (87)

[a] Dihedral angles: $\phi = \tau(CNC^\alpha C)$; $\psi = \tau(NC^\alpha CN)$; $\chi^1 = \tau(NC^\alpha C^\beta S)$; $\chi^2 = \tau(C^\alpha C^\beta SS)$; $\chi^3 = \tau(C^\beta SSC^\beta)$. CS = CS stretch frequencies; SS = SS stretch frequencies.
[b] Ref. 11; w = weak, s = strong.
[c] $XYZ = \chi_1^2\chi^3\chi_2^2$.
[d] $\chi^1\phi,\psi$. $\chi^1 \approx 60°$(H), $\chi^1 \approx -60°$ (C), $\chi^1 \approx 180°$ (N); H, C N are atoms *trans* to S across $C^\alpha C^\beta$ bond; α: −57.4°, −47.5°; β: −138.4°, 135.7°; ε: −80°, 142°.
[e] Potential energy contribution from appropriate bond in parentheses, ≥ 15.
[f] The first number refers to CS (χ_1^2) and the second to CS (χ_2^2).

accord with the observation of the 511 cm^{-1} band. Incidentally, this indicates that the conformations of these S—S bridges in Eb in solution are the same as those in the crystal. The observed band at 524 cm^{-1} is associated with a class, 3, whose range is 521–529 cm^{-1} and whose observed conformations are mainly GT, SD, TT (part of a split mode) and D'T (note that the latter does not agree with the empirical classification[7,8]). The conformation of the S—S bridge in the crystal is in fact GT and the calculated frequency 527 cm^{-1}, is in good agreement with the observed value (see Table 1).

The v(CS) bands are usually weaker and more numerous [since a v(CS) frequency depends only on the structure on its side of the S—S bridge] and are often harder to assign in detail. However, our normal mode analysis of general structures combined with a statistical analysis of 174 C—S conformers in known S—S bridges[6] is helpful in narrowing the possibilities. The obvious band at 657 cm^{-1} should be associated with the two conformations BHβ and GC$\alpha\beta$,[6] where $\alpha\beta$ denotes $-120° < \phi < -60°$ and $0° < \psi < 60°$ (note that the latter structure is not predicted by empirical correlations[7,8]). A weak band near 620 cm^{-1} (as observed) combined with a stronger band near 676 cm^{-1} (which could be masked by the breadth of the 657 cm^{-1} band) would be indicative of a BHα conformation.[6] A band near 700 cm^{-1} could be assigned to B'Cβ, BCβ, GCβ or SCβ conformations.[6] A band near 725 cm^{-1} would be assigned to GCε, TCε or S'Cε.[6] It is possible that v(CS) may contribute near 750 cm^{-1}, since a weak band remains there after tryptophan modification reduces the intensity of the 758 cm^{-1} tryptophan band;[11] if so, GNβ, SNβ, BNβ or TNα conformations would be indicated.[6] It is interesting that in the last

three cases, although χ^2 can vary significantly, χ^1 and ϕ,ψ are almost uniquely determined. Together with the strong inference of structure from other ranges, this emphasizes the observation[6] that detailed information about the $\chi^2\chi^1\phi,\psi$ parameters of an S—S bridge can be obtained from the v(CS) frequencies.

Calculations of the v(CS) modes for the S—S bridges of Eb confirm the above predictions (see Table 1). The 657 cm^{-1} band is assignable to BHβ of cys 55; the *ca.* 700 cm^{-1} band is associated with G'Cβ of cys 3-cys 24; the *ca.* 725 cm^{-1} band arises from G'Cε of cys 17 and TCε of cys 54; and the possible *ca.* 750 cm^{-1} band would be due to GNβ of cys 45. The BHα of cys 60 would contribute to the broadened high-frequency side of the 657 cm^{-1} band, as would the TCε of cys 54 [although the potential energy distribution (PED) of the general structure is inverted compared with the 723 cm^{-1} band[6]]. However, our calculation for cys 60 places the weaker band at 644 (12) cm^{-1}, thus indicating that the position of this band may be sensitive to the details of the structure. Such calculations thus serve to at least associate the observed v(CS) bands with specific residues in the S—S bridges.

γ-II CRYSTALLIN

γ-II crystallin is one of the four fractions of one of the three major structural proteins of the eye lens. It is a chain of 174 amino acid residues, of which seven are cysteine, and its crystal structure has been determined. X-ray analysis of old crystals[13] indicates the presence of a cys 18–cys 22 S—S bridge, whereas in freshly pre-

pared crystals (in the presence of reducing agent) there is no indication of such a bridge,[14] and in fact the SH groups are oriented away from each other.

In this regard, the interpretation of the Raman spectrum has been the subject of controversy. Using apparently similar preparatory procedures, Spector and co-workers[15-17] claimed the presence of one S—S bridge in γ-II crystallin, with a v(SS) band at 511 cm^{-1}, while Yu and co-workers[18,19] did not find any v(SS) band and refuted the presence of an S—S bridge in the native protein. When their protein is incubated at pH 8.2 rather than pH 7.2, a v(SS) band appears at 512 cm^{-1}, presumably due to formation of a cys 18–cys 22 bridge.[19]

We have calculated v(SS) for the molecule containing the cys 18–cys 22 S—S bridge,[13] using a more recently refined structure.[20] The $\phi_1\psi_1\chi_1{}^1\chi_1{}^2\chi^3\chi_2{}^2\chi_2{}^1\phi_2\psi_2$ angles are $-140.1°$, $148.1°$, $-114.6°$, $-98.7°$, $-116.5°$, $173.8°$, $-76.0°$, $96.0°$, $-137.1°$. The computed v(SS) (and PED in SS stretch) are 540(44), 526(15) and 512(31) cm^{-1}. As expected for this conformation,[5,6] v(SS) contributes to more than one band, although in this case the higher PED is in the highest frequency mode. Since the other contributors to these modes are similar and comparably small skeletal angle bends, we would expect the 540 cm^{-1} band to be the strongest of the three.

Do these results shed any light on the conflicting reports about the existence of an S—S bridge in native γ-II crystallin? The clear absence of a v(SS) band[18,19] and the absence of an S—S bridge in freshly prepared crystals[14] strongly indicate that native γ-II crystallin has no disulfide bridge. We must then try to understand why a ca. 511 cm^{-1} band appears in some cases and to what structure(s) it might be due. The 512 cm^{-1} band that appears on pH 8.2 incubation[19] is presumably not due to the formation of a cys 18–cys 22 bridge, since our calculation shows that this should result in a band near 540 cm^{-1}. The 510 cm^{-1} band seen on treatment with 2-mercaptoethanol[18] has been ascribed to the formaton of mixed disulfide bonds. It is interesting, however, that a band at ca. 535 cm^{-1} also appears on such treatment.[18] This may indicate that 2-mercaptoethanol treatment leads to cys 18–cys 22 in addition to mixed disulfide bond formation. Since one preparative procedure involves the use of 2-mercaptoethanol, this could account for the observation of an intramolecular S—S bridge.[15] (The observed 511 cm^{-1} band in this case[16] could not be assigned to a cys 18–cys 22 bridge; these spectra are, however, not good enough to determine if a ca. 540 cm^{-1} band is present.) The calculations thus impose new constraints on possible S—S bridge structures derived from the Raman spectrum.

IMMUNOGLOBULIN

Immunoglobulins (Ig) generally consist of a basic unit of two polypeptide chains, a light (L) and a heavy (H) chain. The L chain has two homology regions, an N terminal variable (V_L) and a constant (C_L), each of which has an intrachain S—S. The H chain of human Ig (IgG) has four homology regions, V_H, C_H1, C_H2 and C_H3, to two of which, V_H and C_H1, V_LC_L is connected

by an interchain S—S bond. Two basic units are connected together by two S—S bonds to form the Ig molecule.

High-resolution x-ray structures have been determined for various fragments: the V_LC_LS—SC_H1V_H region, designated Fab (Fab New[21]), and dimers of the V_L fragment of Bence-Jones (B-J) proteins, V_LC_LS—SC_LV_L (Rhe[22] and REI[23]). In the v(SS) and v(CS) regions, Raman spectra have been obtained[24] of a V_L fragment (Tod), of several B-J proteins (Kob, Nag, Ta) and of IgG. It is therefore of interest to see whether assignments to the above structures can be made on the basis of the observed spectra. We have calculated the modes of the various S—S bridges indicated by the x-ray structures, and these are given for the V_L domains in Table 2 and for the Fab fragment in Table 3.

The Raman spectrum of V_L(Tod) exhibits a v(SS) band at 524 cm^{-1}, and very weak bands at 665 and ca. 750 (shoulder) cm^{-1} (plus a broad weak band at 704 cm^{-1}) that could be assigned to v(CS).[24] The 524 cm^{-1} band belongs to class 3,[5] which is in agreement with the observed SD conformations in the V_L domain (Tables 2 and 3). Since v(SS) modes are split for this conformation and can be found over a large range,[5,6] it becomes particularly important to compare observed frequencies with those calculated for the exact structure. In this case, the observed 524 cm^{-1} band is in best agreement with the predicted v(SS) of the V_L structure of Fab (New), and we therefore suggest this assignment. The predicted v(CS) modes at 665 and 754 cm^{-1} are also consistent with observation. [The only other possible assignments might be to cys 22–cys 89 (Rhe) and cys B23–cys B88 (REI), but in these cases we would expect a split v(SS) band with a strong component near 550 cm^{-1}].

The v(SS) modes of the B-J and IgG proteins are observed in the solid state at[24] Kob 510, 526, Nag 505, 520, 535, Ta 505, 520, 537 and IgG 512, 525 cm^{-1}. For the B-J proteins, the 520–526 cm^{-1} bands are clearly assignable to the V_LS—S bridge, as in Tod. Assuming that the interchain S—S bridge structures in Fab and the B-J protein are similar, the 505–510 cm^{-1} bands can be associated with the interchain S—S bridge (see Table 3). Even though the SB' conformation of this bridge in Fab would indicate a class 1b frequency,[5] the unusually high χ^3 value, leading to a smaller SS stretch force constant,[1] would undoubtedly result in a class 1a frequency. The expected downshift of ca. 5 cm^{-1} for $\chi^3 \approx 166°$[1] indicates that Nag and Ta adopt the Fab structure. This suggests that perhaps in Kob and IgG the χ^3 angle of the SB' conformation is closer to normal. The C_L domain contains the remaining S—S bridge, and we can therefore assign the 535 (Nag) and 537 (Ta) cm^{-1} bands to this structure. (The absence of such a band in the spectra of IgG and Kob, although it appears in the latter at 538 cm^{-1} in solution at 18 °C at pH 6.0,[24] may reflect the fact that such class 4 bands are often split[5] and therefore the weaker components may not be seen except in high-quality spectra). Since our calculated v(SS) frequencies have agreed with observed bands to within ca. 5 cm^{-1} in molecules of known structure,[4] the ca. 15 cm^{-1} discrepancy with the predicted v(SS) mode of C_L in Fab (551 cm^{-1}) suggests that the structure of the C_L S—S bridge in the B-J proteins is different from that in Fab.

Table 2. Disulfide bridge frequencies (in cm⁻¹) of V_L fragments

τ^a	Rheᵇ Cys 22–Cys 89 SGDᵈ $N\beta^e$	Rheᵇ Cys 22–Cys 89 SGDᵈ $H\varepsilon$	REIᶜ Cys A23–Cys A88 D'G'S' $C\beta$	REIᶜ Cys A23–Cys A88 D'G'S' $H\varepsilon$	REIᶜ Cys B23–Cys B88 SGD $N\beta$	REIᶜ Cys B23–Cys B88 SGD $H\varepsilon$
ϕ	−144.2	−106.3	−140.3	−105.9	−145.4	−99.3
ψ	118.4	146.5	125.7	148.3	123.0	148.3
χ^1	171.2	53.9	−94.2	77.1	180.0	59.4
χ^2	122.6	162.8	−153.1	−133.3	123.3	158.7
χ^3	87.3		−91.0		81.1	
CS			784 (29)ᶠ			
	756 (46)				755 (59)	
			703 (26)			
			671 (22)			
		664 (55)				663 (58)
			658 (72)			
SS	556 (33)		547 (42)		552 (39)	
	515 (32)		521 (31)		518 (35)	

a Dihedral angles: $\phi = \tau(\text{CNC}^a\text{C})$; $\psi = \tau(\text{NC}^a\text{CN})$; $\chi^1 = \tau(\text{NC}^a\text{C}^\beta\text{S})$; $\chi^2 = \tau(\text{C}^a\text{C}^\beta\text{SS})$; $\chi^3 = \tau(\text{C}^\beta\text{SSC}^\beta)$. CS = CS stretch frequencies; SS = SS stretch frequencies.
b Ref. 22. This is a symmetric dimer.
c Ref. 23.
d $XYZ = \chi_1{}^2\chi^3\chi_2{}^2$.
e $\chi^1\phi,\psi$. $\chi^1 \approx 60°$ (H), $\chi^1 \approx -60°$ (C), $\chi^1 \approx 180°$ (N); H, C, N are atoms *trans* to S across C^aC^β bond; α: −57.4°, −47.5°; β: −138.4°, 135.7°; ε: −80°, 142°.
f Potential energy contribution from appropriate bond in parentheses, ≥15.

The effects of acid and temperature denaturation on the B-J protein Kob have also been studied.[24] The ν(SS) bands of Kob in solution at pH 6.0 and room temperature are at 516, 525 and 536 cm⁻¹. Whereas the latter two frequencies are close to those of Kob in the solid state, indicating similar V_L and C_L S—S bridge conformations, the large increase in the first frequency suggests that the interchain S—S bridge may have a different conformation in solution to that in the solid state. Its class 2 frequency[5] would be consistent with a change from SB' with an unusual χ^3 to perhaps TB' or DG' with a more normal χ^3. At pH 1.3 these bands are

Table 3. Disulfide bridge frequencies (in cm⁻¹) of IgG Fab (New)ᵃ

τ^b	V_L Cys L22–Cys L89 SGDᶜ $N\beta^d$	V_L Cys L22–Cys L89 SGDᶜ $H\varepsilon$	C_L L136–L195 TGT $N\beta$	C_L L136–L195 TGT $N\varepsilon$	V_H H22–H95 TGT $N\beta$	V_H H22–H95 TGT $N\beta$	C_H H144–H200 TG'D' $N\beta$	C_H H144–H200 TG'D' $N\varepsilon$	Interchain L213–H220 SGB' N^e	Interchain L213–H220 SGB' H^e
ϕ	−131.1	−114.8	−137.3	−107.6	−133.1	−129.8	−149.6	−94.6	117.8	−18.0
ψ	83.6	129.4	99.7	110.1	121.3	85.4	95.3	106.9	−50.5	135.3
χ^1	154.5	56.5	−169.0	−179.9	176.0	156.5	−126.3	109.3	156.2	16.7
χ^2	118.9	155.8	179.4	−175.6	171.9	−167.6	−171.9	−152.0	111.6	−76.9
χ^3	110.5		−94.4		−66.1		−79.7		166.8	
CS					783 (50)ᶠ					
			775 (61)		777 (60)		774 (56)			
			763 (62)							
	754 (36)								742 (28)	
	698 (22)						701 (20)			
									689 (43)	
							673 (36)			
	665 (59)									
SS	557 (21)		551 (68)		549 (65)					
							544 (74)			
	526 (56)									
									510 (80)ᵍ	

a Ref. 21.
b Dihedral angles: $\phi = \tau(\text{CNC}^a\text{C})$; $\psi = \tau(\text{NC}^a\text{CN})$; $\chi^1 = \tau(\text{NC}^a\text{C}^\beta\text{S})$; $\chi^2 = \tau(\text{C}^a\text{C}^\beta\text{SS})$; $\chi^3 = \tau(\text{C}^\beta\text{SSC}^\beta)$. CS = CS stretch frequencies; SS = SS stretch frequencies.
c $XYZ = \chi_1{}^2\chi^3\chi_2{}^2$.
d $\chi^1\phi,\psi$. $\chi^1 \approx 60°$ (H), $\chi^1 \approx -60°$ (C), $\chi^1 \approx 180°$ (N); H, C, N are atoms *trans* to S across C^aC^β bond; α: −57.4°, −47.5°; β: −138.4°, 135.7°; ε: −80°, 142°.
e Unusual ϕ,ψ, for L chain, $\psi = \text{NC}^a\text{C(O)O}$ for H chain.
f Potential energy contribution from appropriate bond in parentheses, ≥15.
g Calculated with force field for $\chi^3 \approx 90°$. See text for discussion.

found at 516, 525 and 533 cm^{-1}, indicating a small change (if any) only in the S—S bridge conformation in the C_L region. On increasing the temperature from 8 to 44 °C, the main changes are a shift of the 516 cm^{-1} band to 513 cm^{-1}, the essential disappearance of the 525 cm^{-1} band and the near constancy of the high-frequency band (at 543 cm^{-1}). The main effect could be explained by the V_L S—S bridge taking on a range of conformations, so that no well defined structure predominates. This would be consistent with the observation[25] that temperature-dependent changes occur mainly in the V_L domains.

CONCLUSIONS

Normal mode calculations of disulfide bridge frequencies provide more specific information about this structure from Raman spectra than is possible from empirical correlations.[7,8] This is illustrated here by such analyses of the spectra of erabutoxin b, γ-II crystallin and fragments of the IgG molecule. Not only can observed bands be associated with well defined confor-mations, including the ϕ,ψ values of adjacent peptide groups, but calculations based on known x-ray structures can reveal whether S—S bridges in related molecules or different physical states adopt these conformations.

For Eb, we are able to assign observed $v(CS)$ bands to specific components of the S—S bridges. In the case of γ-II crystallin, we can show that the ca. 511 cm^{-1} $v(SS)$ band observed in some preparations does not arise from the cys 18–cys 22 bridge found in the structure of old crystals. For the IgG fragments, we can determine whether or not observed Raman bands correspond to bridge structures obtained from x-ray studies, and also suggest the likely structural changes resulting from acid and temperature denaturation. Such approaches are likely to be equally helpful in studying details of disulfide bridge structures in other proteins.

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