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A gentle vapor-diffusion technique for cross-linking of protein crystals for cryocrystallography

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Abstract

Protein crystals have been cross-linked by a gentle technique whereby glutaraldehyde is introduced by vapor diffusion into the crystallization droplet containing the crystals. Diffraction analyses of crystals of three different proteins show that cross-linking prevents, in a large part, the lattice disorder normally observed on rapid cooling of these crystals. The diffraction results suggest that this cross-linking procedure, performed as a simple extension of the standard vapor-diffusion crystallization experiment, may generally aid in the cooling of fragile protein crystals for which standard procedures of cryopreservation prove inadequate.

1. Introduction

Cryocrystallography is often a critical tool in the structure determination of proteins and other macromolecules. All protein crystals are sensitive to radiation damage, and this sensitivity can be unmanageable with derivative forms of the crystals or with fragile crystals. Diffraction quality of these crystal types deteriorates rapidly, within minutes of being exposed to the high intensity of a synchrotron beam. Under these circumstances, low-temperature diffraction, first reported by King (1958) and Low *et al.* (1966), offers the best way to preserve the intrinsic quality of the crystal. Techniques for rapid cooling of crystals have proven straightforward, and many crystals in the appropriate cryoprotectant have been cooled to cryogenic temperatures without significant loss of lattice integrity (reviewed by Hope, 1990; Watenpaugh, 1991; Rodgers, 1994). Successful cooling of crystals depends to a large extent on optimizing the composition of the cryoprotectant solution with respect to conditions of pH, the buffer, the salt, the nature of the organic cryoprotectant, and its final concentration in the solution (Rodgers, 1997, and references therein). There are nonetheless many fragile crystals (three types are cited here) for which an appropriate cryoprotectant cannot be found, and these crystals suffer irreversible damage on cooling. Disordering of the lattice leads to imperfections in the diffraction pattern that include, but are not limited to, unacceptable mosaic spread, fractured lattice lines, anisotropy, or nonisomorphism across the crystal.

In one approach to this problem, Haas & Rossmann (1970) used glutaraldehyde cross-linking (Quiocho & Richards, 1964) followed by soaking of the cross-linked crystals in glycerol to prevent lattice damage observed on cooling. Cross-linking tends to strengthen the internal structure of crystals and results in a marked increase in crystal strength and complete insolubility of the crystal in water (Quiocho & Richards, 1964). The method has been used extensively to stabilize crystals for X-ray diffraction and for procedures such as exchange of solvent, preparation of heavy-atom derivatives, or the introduction of ligands, all of which might otherwise dissolve or disorder fragile crystals.

Despite the advantages conferred by cross-linking, it has found only occasional use in recent years. Many crystals are disordered on cross-linking and there is a variability among experiments. In low-temperature applications, Hass (1968) and Cucka *et al.* (1970) reported that cross-linking led to no reduction in mosaicity caused by cooling. A problem with the use of glutaraldehyde is that it is often difficult to establish the required intermolecular cross-links without loss of diffraction quality. This is probably because cross-linking is generally performed with an excess of reagent and under a broad range of pH and reaction conditions, by which the chain lengths of the cross-links vary, depending on the proportions of a large number of conjugated oligomers present in the solution (reviewed by Peters & Richards, 1977).

The chemistry of the glutaraldehyde reactions, however, was not understood until Monsan *et al.* (1975) reported the effect of pH on the structure of glutaraldehyde in solution and defined the mechanism and products of its reaction with amino acids and protein lysine. They showed that, at 298 K, the commercial reagent obtained as an aqueous solution of 25% glutaraldehyde, at pH ~3, contains 3% glutaraldehyde, 79% water and 18% hydrated molecules in the form of an unstable cyclic hemiacetal and its oligomers ($n \geq 2$) in rapid reversible temperature-dependent equilibria with one another. As the pH of the solution is increased

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within the range 4–8 and above, the free aldehyde undergoes base-catalyzed aldol condensation followed by dehydration [equation (1) below] and forms α,β-unsaturated oligomers [equation (2)], the value of n increasing slowly as the reaction progresses. The more basic the solution, the more susceptible will be the aldehyde to condensation. Monsan *et al.* (1975) also found that the two predominant products of the condensation reactions are the conjugated dimer ($n = 0$) and trimer ($n = 1$) forms of glutaraldehyde.

$$(n+2)\ \mathrm{OHC{-}(CH_2){-}(CH_2)_2{-}CHO} \rightarrow \mathrm{OHC{-}(CH_2)_3{-}CH{=}C(CHO){-}(CH_2)_2{-}CHO} \quad (1)$$

$$\rightarrow \mathrm{OHC{-}CH_2{-}(CH_2)_2{-}CH = [C(CHO){-}(CH_2)_2{-}CH{=}]_n\ C(CHO){-}(CH_2)_2{-}CHO} + n\mathrm{H_2O} \quad (2)$$

At pH 3, the ε-amino group of lysine reacts with free glutaraldehyde and forms predominantly the Schiff base linkage in analogy with the mechanism of Cordes & Jencks (1962). At pH above 4, Schiff base formation decreases sharply with pH, until at pH 7 lysines react preferentially (~90%) with the conjugated aldehyde groups of reactive oligomers and form the resonance-stabilized imine. A pK_a of 4.63 ± 0.05 for the overall formation of the conjugated imine can be calculated from available data (Fig. 2 of Monsan *et al.* 1975) by measuring the peak areas corresponding to acid-stable product formed in the pH range 2–10.5 and fitting the results to the equation HABELL, $\log y = \log(c/l + H/K_a)$, with the Fortran program of Cleland (1979). The doubly conjugated lysine derivative has been identified as the acid-stable and principal product found in all cross-linked crystals examined (reviewed by Peters & Richards, 1977).

The properties of glutaraldehyde summarized above were used in the present studies to develop a gentle method for selective cross-linking of fragile crystals for low-temperature diffraction measurements. The volatile nature of glutaraldehyde allowed us to introduce the free aldehyde by vapor diffusion from a separate reservoir into crystallization droplets. The resulting cross-linked crystals permitted analysis of crystal structure data in three cases where cryopreservation of the crystal could not be achieved through standard procedures for rapid cooling.

2. Materials and methods

2.1. *Crystals*

All crystals were grown at 293 K in hanging droplets by the vapor-equilibrium method. Crystals of a selenomethionyl two-domain fragment of neural *N*-cadherin were grown at low ionic strength in the presence of 2 m*M* $CaCl_2$ and 1 m*M* uranyl acetate after a pH shift to around 5, maintained by 50 m*M* Na acetate buffer (Tamura *et al.,* 1998). Moloney murine leukemia virus (MMLV) reverse transcriptase-substrate DNA co-crystals were grown from 100 m*M* Hepes, 75 m*M* NH_4Cl and 14% PEG 3350, at pH 7.5. Before cross-linking, crystals were derivatized by introduction of 2.5 m*M* mercuric acetate into the crystallization droplet and soaking (Sun *et al.*, 1998). A ternary complex composed of a truncated form of gp120 from HIV-1, the N-terminal two domains (D1D2) of CD4, and a Fab from a human neutralizing antibody, was crystallized from 50 m*M* citrate, pH 6.3, 10% PEG 5000 (monomethyl ether) and 10% isopropanol (Kwong *et al.*, 1998).

2.2. *Reagent*

Purified (grade I) glutaraldehyde in 25% aqueous solution, at pH 3, held under argon in sealed glass ampoules, was obtained from Sigma Chemical Co., stored at 193 K, and used once. After warming to room temperature, the reagent was transferred to a 1.5 ml Eppendorf tube (which was then tightly capped to prevent evaporation) and held at ambient temperature. Glutaraldehyde is toxic and precaution was taken against exposure to the reagent by conducting the cross-linking reactions in a chemical fume hood. At the end of the experiments, glutaraldehyde was precipitated by adding 0.1 ml of 1 *M* NaOH to all solutions containing the reagent, and the insoluble products were discarded.

2.3. *General procedure for cross-linking*

Cross-linking reactions were performed at room temperature in a Linbro plate reservoir containing 0.5–1 ml of the precipitant solution used in crystallization and a micro-bridge (Hampton Research), which serves as an isolated reservoir for the reagent. In the general procedure, an aliquot (2–5 µl) of 25% glutaraldehyde, at pH 3, is placed in the well of the microbridge, and the cross-linking reaction is immediately initiated by sealing a cover slip containing one or more crystals in a hanging droplet (1.5–2 µl) over the reservoir (Fig. 1). The reaction is allowed to proceed for 30–60 min. At the end of the time period, the cover slip is moved away from the

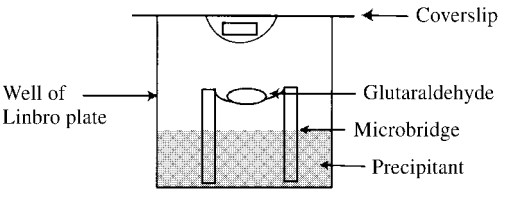

Fig. 1. Scheme for vapor diffusion of glutaraldehyde for cross-linking of protein crystals. The drawing represents one well of a Linbro plate. The reservoir solution used for crystallization, the well of a microbridge containing a sitting droplet of fixed volume (2–5 µl) of aqueous glutaraldehyde, pH 3, and the cover slip containing a crystallization droplet are indicated.

cross-linking reservoir and placed over a new reservoir containing fresh precipitant solution without glutaraldehyde. This provides the advantage that crystals can be easily handled under the microscope. If the cross-linked crystals are to be immediately prepared for freezing, they may be mounted directly from the droplet and cryoprotected without the need to remove excess reagent. Otherwise, the reaction is promptly terminated by removing excess reagent with addition of 5–10 µl of the appropriate stabilizing solution (without cryoprotectant) to the crystals, followed by washing three times with 30 times the droplet volume. The crystals are placed over a reservoir containing fresh stabilizing solution without cryoprotectant and stored.

2.4. *Comments on the procedure*

None of the crystals described here exhibited yellow color on cross-linking. The appearance of increasing yellow color reflects the incorporation of chromophoric long-chain conjugates in the protein, and such crystals are often found to be disordered (Quiocho & Richards, 1964). All other variables remaining constant, at a fixed amount (concentration × volume) of glutaraldehyde in the bridge reservoir, the extent of cross-linking was proportional to elapsed time during the 60 min period. Cross-linking was found to be highly reproducible among crystals of the same crystal type, as judged by diffraction quality of the cross-linked crystals. Success in selective cross-linking of molecules in the lattice, however, varies with the protein crystal under study (see §4). Therefore, it may be found necessary to alter the extent of cross-linking by shortening or lengthening the time of exposure to the reagent, changing pH, or modifying the concentration of glutaraldehyde in the bridge reservoir (2–5 µl droplet). In the latter case, it is important that the stock reagent is diluted with 0.001 *N* HCl (pH ∼3); otherwise, there may be little free aldehyde in the solution, due to the condensation reaction. Several crystals in a droplet can be conveniently screened for optimal cross-linking by removing a crystal at various time intervals and assaying solubility in water. Cross-linked crystals are apt to dissolve very slowly, so crystals are observed over a period of several days to evaluate crystal solubility. Those reaction conditions bringing the crystal just into the insoluble range usually lead to near-optimal cross-linking.

2.5. *Cryoprotection and rapid cooling of cross-linked crystals*

Cross-linked crystals were prepared for cooling, by using the appropriate cryoprotectant and procedures previously optimized for each crystal type. *N*-Cadherin crystal derivatives were flooded with 5 µl of crystallization buffer and mounted in a cryoloop (Teng, 1990) directly from the droplet. The mounted crystal was placed in ∼2–3 µl of a crystallization buffer containing 25% (*w*/*v*) sucrose and equilibrated with the solution for 15 min at room temperature. This step was repeated with ∼3 µl of crystallization buffer containing 50% (*w*/*v*) sucrose, then external solvent was removed by streaking the crystal through a droplet (500 µl) of Paratone-N oil (Exxon). The loop-mounted crystal was flash-cooled by placing the crystal directly into a nitrogen gas cryostream (110 K) (*cf.* Tamura *et al.*, 1998). Derivative crystals of reverse transcriptase were mounted directly from the cross-linking droplet and equilibrated over a period of 2 h by serial transfers of the crystals into stabilizing solutions made with the heavy-atom cation (2.5 mmol l^{-1}) and increasing proportions (1–20% *v*/*v*) of ethylene glycol. The loop-mounted crystals were cooled by placing them directly into the nitrogen cryostream (100 K) (Sun *et al.*, 1998). gp120 co-crystals were transferred directly from crystallization droplets into a stabilizing solution that contained 10% (*v*/*v*) ethylene glycol and equilibrated with the solution for 10–15 min at room temperature. The crystals were transferred to Paratone-N oil (500 µl) and external solvent was carefully removed, as described by Kwong & Liu (1999). The crystal, mounted in a small cryoloop, was placed directly in the nitrogen cryostream and cooled to 100 K.

2.6. *Diffraction analysis*

Diffraction data were collected with synchrotron radiation at a wavlength of ∼1 Å at beamline X4A at the National Synchrotron Light Source, Brookhaven National Laboratory. The diffraction quality of crystals of each crystal type was determined from 1° oscillation images of the crystal in two directions recorded on Fuji BASIII phosphor imaging plates, with intensities measured with a BAS2000 scanner (Fuji). Image-plate patterns (sufficient data to represent the entire pattern) were analyzed with the image-processing program *HKL* (Otwinowski & Minor, 1997) to assess cell dimensions, diffraction limit and crystal mosaicity. The diffraction limit was estimated by the position of the highest resolution diffraction spot visible in the image-plate display program of *HKL*. In our experience, this value corresponds to the limit at which $I/\sigma(I) \geq 2$. Crystal mosaicity was determined by *SCALEPACK* (Otwinowski & Minor, 1997) post-refinement and *HKL*, and mosaicity reported here refers to the full width of the rocking curve at zero maximum, as reported by the *HKL* program. The unit-cell parameters, diffraction limit and crystal mosaicity of a single cross-linked crystal of each crystal type at 100 K were also obtained from analysis of complete data sets, which were ultimately used for the structure determinations detailed in the separate publications cited below.

Table . *Effect of cross-linking on the diffraction properties of flash-cooled crystals of an N-cadherin fragment, a reverse transcriptase–DNA complex, and a gp120 ternary complex*

		Without cross-linking		With cross-linking	
Protein crystal	Temperature (K)	d_{min} (Å)	Mosaicity† (°)	d_{min} (Å)	Mosaicity‡ (°)
Two-domain *N*-cadherin derivative	298	2.9	0.4		
	100	2.9	>2	2.9	0.7
Reverse transcriptase derivative	298	1.9	0.45		
	100	1.9	1.5–2§	1.9	0.45
		>15 Å (>11%) decrease of *c* cell dimension		6 Å (4.4%) decrease of *c* cell dimension	
gp120 ternary complex	298	2.7	0.5		
	100	2.7	>5	2.2	<1
		Broad split spots		Compact single spots	

† Mosaicity refers to the full width of the rocking curve at zero maximum, as reported by the *HKL* program.
‡ Diffraction limit and crystal mosaicity represents the mean of measurements obtained from four or five independent samples of crystals of each crystal type. The diffraction limit and mosaicity of one sample of each crystal type were obtained from a complete data set collected from a single frozen crystal. Variation from the mean was below the level of statistical significance.
§ The mosaicity range indicates the variation of at least three independent samples.

3. Results

The effects of cross-linking on the low-temperature diffraction properties of crystals of a selenomethionyl *N*-cadherin fragment (Tamura *et al.* 1998), a DNA complex of MMLV reverse transcriptase (Sun *et al.*, 1998) and a gp120 ternary complex (Kwong *et al.*, 1998) are summarized in Tables 1 and 2. Variations in the measurements of diffraction limit and crystal mosaicity among cross-linked crystals ($n = 4$–5) of each crystal type were below the level of statistical significance.

3.1. *Two-domain N-cadherin fragment*

Diffraction measurements of derivative crystals (space group *I*422) of two-domain *N*-cadherin containing a heavy atom showed that the crystals are capable of diffracting to 2.9 Å and with 0.4° mosaicity at room temperature. However, the crystals underwent rapid radiation damage, and diffraction quality deteriorated after a few degrees of data collection. Diffraction data collected from frozen crystals proved impossible to analyze, due to a large increase in mosaicity (>1.6°) on cooling that led to broadening of the rocking curve to >2° (Table 1). To stabilize the lattice during rapid cooling, we used cross-linking. *N*-Cadherin crystals contain four lysines in the asymmetric unit, so only a few cross-links between molecules would be expected. Derivative crystals were cross-linked at pH 5.0 by exposure to 50 µl of aqueous reagent (5–25%) for 30–60 min (Table 2). Under these conditions, cross-linking appeared to be complete at all concentrations of glutaraldehyde tested. Each of the small delicate crystals (50 × 50 × 10 µm) proved to be remarkably stable and completely insoluble in water; presumably this is due to the formation of cross-links between molecules in the lattice. Cross-linked crystals were mounted directly from the droplet, cryoprotected and flash-cooled as described above (§2). Crystals were monitored for quality of diffraction in terms of changes in intensity distribution, unit-cell parameters, diffraction limit and crystal mosacity (Tables 1 and 2). Cross-linked crystals in the frozen state gave the same diffraction patterns as normal (unmodified) crystals at room temperature; no significant differences were observed in intensity distribution, cell parameters or diffraction limits. Crystal mosaicity increased 0.3°, an increase fivefold lower than that observed on cooling of unmodified crystals (>1.6°). The reason for the modest increase in mosaicity (0.3°) observed with cross-linked crystals cannot be explained. Nonetheless, the mosaicity of the cross-linked derivative (0.7°) was in the range permitting complete data collection and structure analysis (Tamura *et al.*, 1998).

3.2. *MMLV reverse transcriptase complex*

Derivative crystals of reverse transcriptase diffracted with high resolution and low mosaicity at room temperature (Table 1), but the crystals sustained radiation damage, and data collection was limited to 2.8 Å. Without cross-linking, it had been technically impossible to freeze derivative crystals and collect usable diffraction data. A marked shrinkage (11%) in the *c* dimension was coupled with an increase in *c*-axis mosaicity, suggesting that not all cells in the crystal shrank by the 15 Å change calculated from the final *c* cell dimension. The variation in shrinkage across the crystal resulted in a discrete diffraction pattern superimposed upon a diffuse fiber-like pattern and a very large (>2°) mosaic spread, which made these crystals unusable for accurate data collection.

In a modified version of the diffusion method, derivative crystals were cross-linked by depositing a droplet (2 µl) of 25% glutaraldehyde on the coverslip next to the crystal droplet. After exposure to the reagent for 2 h, the crystals became completely insoluble in water. Cross-linked crystals were mounted directly from the droplet and were cryocooled as described in §2.

Table . *Properties of cross-linked crystals of each crystal type at low temperature and conditions of cross-linking*

Crystal	Salt/pH	Space group and unit-cell parameters†	Solvent content, calculated (%)	Lys per asymmetric unit	Glutaraldehyde treatment	Cryoprotectant (concentration)‡
Two-domain *N*-cadherin§	50 m*M* NaOAc, pH 5.0	*I*422, $a = b = 99.0$, $c = 136.1$ Å	57.4	4	50 µl 10%, 30–60 min	Sucrose (50% *w*/*v*) and Paratone-*N*
Reverse transciptase DNA complex¶	100 m*M* Hepes, 75 m*M* NH_4Cl, 14% PEG 3350, pH 7.5	$P2_1$, $a = 61.1$, $b = 38.4$, $c = 129.8$ Å, $\alpha = \gamma = 90$, $\beta = 100.6°$	~45	28	2 µl 25%, 2 h	Ethylene glycol (20% *v*/ *v*)
gp120 ternary complex	50 m*M* Na citrate 10% PEG 5000, (monomethyl ether), pH 6.3, 10% isopropanol	$P222_1$, $a = 71.6$, $b = 88.1$, $c = 196.7$ Å	58	62	20 µl 1%, 60 min	Ethylene glycol (10% *v*/ *v*) and Paratone-*N*

† Unit-cell parameters indicate the mean of four or more independent samples of each crystal type. Variation in the measurements was below the level of statistical significance.
‡ Concentration refers to the proportion (*w*/*v*) of cryoprotectant in the stabilizing solution. The stabilizing solution was never diluted by addition of the cryoprotectant.
§ A derivative crystal of the selenomethionyl *N*-cadherin fragment, containing uranyl acetate and $CaCl_2$.
¶ A derivative crystal containing mercuric acetate.

Diffraction patterns obtained from crystals at 100 K were found to be sharp and similar in quality to those of unmodified crystals at room temperature. Even though a significant (4.4%) shrinkage in the *c* cell dimension was observed, the distortion was not associated with an increase in crystal mosaicity. The crystals diffracted with the same low mosaicity (0.45°) and high resolution limit (1.9 Å) as unmodified crystals at room temperature (Table 1).

In agreement with the results of earlier studies (Quiocho & Richards, 1964), analysis of crystal structure data obtained from a single cross-linked crystal at 100 K indicated that the presence of cross-links between protein molecules in the lattice led to no detectable differences in the structure of the crystalline domains of the complex, even at the level of high resolution (1.9 Å) (M. Georgiadis, personal communication).

3.3. *HIV-1 gp120 ternary complex*

gp120 co-crystals (space group $P222_1$) initially diffracted with 2.7 Å resolution and 0.5° mosaicity at room temperature (Table 1), but the fragile crystals proved sensitive to radiation damage, and diffraction quality was rapidly lost. Cooling the crystals invariably led to broadened and multiply split reflections, making diffraction data greater than 3 Å unusable. In contrast to crystals of *N*-cadherin and reverse transcriptase, when gp120 crystals were cross-linked enough to make the crystal completely insoluble in water, the crystals lost diffraction quality. A problem particular to cross-linking gp120 crystals is the requirement of isopropanol in the crystallization process, as alcohol–water mixtures are known to increase the rate of dehydration (Cordes & Jencks, 1962). Therefore, progressively milder conditions of cross-linking were tried by diluting the glutaraldehyde reagent in the bridge reservoir and shortening the reaction time. With less extensive cross-linking, the size and shape of diffraction spots improved and resolution increased. When prepared at pH 6.3 with 20 µl of 1% aqueous glutaraldehyde in the bridge reservoir and a reaction time of 60 min, gp120 crystals were sufficiently cross-linked to withstand the stress of cooling. The fragile crystals became mechanically harder and appeared to be insoluble. However, when the crystal was placed in water overnight, the interior of the crystal dissolved, and the remaining crystalline shell showed that the crystal was stably cross-linked only near the surface. The surface-cross-linked crystals, however, diffracted with single compact reflections extending to $d_{min} = 2.2$ Å, a significantly higher diffraction limit than that of the crystal at room temperature (Table 1). Crystal mosaicity increased by 0.5° on cooling, but the increase was ninefold lower than that observed with the unmodified crystal. Even though the mosaicity of the cross-linked crystal was relatively high (~1 Å), data collection and structure analysis could be achieved.

4. Discussion

The results presented here and summarized in Tables 1 and 2 illustrate the potential of cross-linking as a valu-

able adjunct to standard cryopreservation procedures, for use when it is otherwise not possible to freeze fragile protein crystals without disordering the lattice. The diffusion method for cross-linking is simple and is highly reproducible from crystal to crystal and from laboratory to laboratory. Cross-linking is initiated with virtually no disturbance to the crystal, as the salt, precipitant and buffer conditions of crystallization are maintained. To increase the selectivity of cross-linking to a few intermolecular bonds between protein molecules in the lattice, we tried as far as possible to reduce nonspecific cross-linking with other areas of the protein by keeping the molecular ratio of glutaraldehyde to crystal protein low (∼1). Under these conditions, the rate of cross-linking appears to be limited by the concentration of free aldehyde in the crystal droplet, at least during the early part (15–30 min) of the reaction, when specific intermolecular cross-links are generally observed.

Ultimately, the success of cross-linking depends on the specific crystallographical positioning of lysines and the total number of lysine groups in the asymmetric unit. One concern is with proteins containing only a small number of lysines (*e.g.* less than five): no lysine may be suitably oriented and within proper distance for cross-linking between molecules in the lattice. One of the crystal types examined, namely two-domain *N*-cadherin, contains only four lysines per molecule and as it is in space group *I*422, contains only one molecule per asymmetric unit; nonetheless, glutaraldehyde cross-linking worked very well with this crystal. Inspection of the crystal lattice in the refined structure of the cross-linked derivative (Tamura *et al.*, 1998) showed three lysine pairs to be juxtaposed between symmetry-related mates in the unit cell. The ε-NH_2 groups of one lysine pair were situated 9 Å apart, well positioned to accommodate a five-carbon cross-link. Estimated from δ-C positions, the other two symmetry-related pairs were separated by 14 Å, a distance requiring a minimum cross-link of ten C atoms in length (L. Shapiro, personal communication). Cross-linking presumably takes place in two steps: the first is the formation of a reversible Michaelis complex, and the second, the irreversible reaction covalently linking the aldehyde reagent to the protein. In this way, the large number of conjugated oligomers present during the cross-linking reaction could use their inherent binding properties to promote a link of optimal length. Even though none of the cross-links could be directly observed in the *N*-cadherin structure refined to 3.4 Å resolution, the remarkable stability of the lattice on cross-linking provides convincing evidence for the existence of one or more of the required cross-links between molecules. The question whether cross-linking has fixed or altered the side-chain conformation in the crystal cannot be answered until structure information from the unmodified crystal is available.

Cross-linking was used here to strengthen the crystal lattice against the mechanical stress of freezing. However, it is also potentially useful in other situations where fragile crystals undergo mechanical stress, for example in transfer to stabilizing solutions of very different buffer or salt composition, or in the preparation of heavy-atom derivatives or of substrate or other ligand complexes. The diffusion method of cross-linking described here may prove valuable for these and other purposes.

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