Vol. 80, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1978 Pages 886-890

INTERMOLECULAR CROSS-LINKING OF A PROTEIN CRYSTAL - ACID PROTEASE FROM ENDOTHIA PARASITICA - IN 2.7 M AMMONIUM SULFATE SOLUTION

C. Wong, T.J. Lee, T.Y. Lee, T.H. Lu and C.S. Hung
National Tsing Hua University, Hsinchu, Taiwan, China

Received October 4,1977

Summary: A workable procedure for cross-linking of a protein crystal-acid protease from Endothia parasitica - in 2.7 M ammonium sulfate solution with bifunctional reagent glutaraldehyde has been found. Using the cross-linking technique, we have made one good quality heavy atom derivative of uranyl into three or even more isomorphous derivatives for this particular protein.

Previous success in cross-linking of a protein crystal with bifunctional reagent glutaraldehyde (1,2) (Carboxypeptidase-A is a good example of its application to X-ray structural determination (3)) has been limited to those crystals grown from mother liquid containing no ammonium sulfate. However, the majority of protein crystals have been grown from rather concentrated ammonium sulfate solutions, but in such solutions the heavy atom binding with protein would be suppressed in the presence of ammonium ion (4,5). This has been the main obstacle which has slowed down or even prohibited the determination of many protein crystals grown from ammonium sulfate solution. The alternative to bypass this obstacle is to change mother liquid, (α-chymotrypsin from 2M $(NH_4)_2SO_4$ to either 3.6M NaH_2PO_4 or 2.2M $MgSO_4$(4)) or to regrow it from non-ammonium sulfate solutions (erabutoxin b, using Na_2SO_4 solution instead (6)). However, most of the time, great difficulty was involved. If a protein crystal grown from ammonium sulfate solution is cross-linked, the crystal would be stable in many non-ammonium salt solutions or even pure water, and in broader range of pH. These properties are

0006-291X/78/0804-0886$01.00/0

advantageous in preparing heavy atom derivatives.

Over three years ago, we started work on the x-ray structural investigation of an acid protease from fungus Endothia parasitica (7,8,9). In the course of our study, we have tried various approaches to obtain good heavy atom derivatives. One approach which we found to be very useful is to cross-link this protease crystal with glutaraldehyde directly from its mother liquid in 2.7M $(NH_4)_2SO_4$ solution. We have since worked out a fool-proof procedure for cross-linking this protein crystal. We hope that our method with some modification may apply to other protein crystals grown from ammonium sulfate solutions.

The procedures are:

1. The crystals of average size 0.5x1.0x0.25mm are originally in mother liquid (2.7M $(NH_4)_2SO_4$, 0.1M NaH_2PO_4, pH 6.5). To one ml of mother liquid, 0.4ml of 25% glutaraldehyde is added. The solution immediately becomes milky, and gradually turns to reddish brown in about 15 minutes. After one hour, the solution becomes dark brown and a thin layer of gummy precipitate is formed. All the dark brown liquid is drawn out with a pipette and the crystals are washed several times with deionized water (all wihin 5 minutes).

2. The partilly cross-linked crystals, light yellow in color, are then placed in salt-free 10% glutaraldehyde solution overnight (15-24 hours).

3. The strongly cross-linked crystal then are golden brown in color, and are relatively unbreakable. These crystals are washed several times with water and are stored in water until ready for use. They can be stored this way for about 20 days, after which they will decay rapidly.

Step 1 is most important in obtaining good cross-linked crystals. It is found by trial and error. The condition requires that initially when the glutaraldehyde is introduced into the mother liquid, the sudden dilution of the mother liquid from 2.7M to 2M $(NH_4)_2SO_4$ would not break up the crystals. Also, the glutaraldehyde used is about 7% which is much more concentrated than the previously used 1% in protein crystals grown from solutions containing no ammo-

Table 1

	a	b	c	β	Extend of diffraction pattern	Decay time†	specific activity for milk clotting††
Cross-linked crystal	54.1(1)A	74.5(1)A	45.9(1)A	110.5(1)°	< 2.5A	70	2-5 u/mg
Untreated crystal	53.6(1)	74.1(1)	45.7(1)	110.0(1)°	< 2.5A	36	100 u/mg
UA	54.0(1)	74.2(2)	45.9(1)	110.5(1)°	< 2.5A	26	
UB	53.8(1)	74.3(1)	45.8(1)	110.3(1)°	< 2.5A	26	
UC	54.1(1)	74.2(1)	45.8(1)	110.5(1)°	< 2.5A	26	

† Decay time are expressed in the number of hours for decrease of 10% intensity as exposed to 48KV, 25mA CuKα X-ray.

†† According to Hagemeyer et al (8).
The R-factor between cross-linked and untreated crystal is 0.10 and is defined as:

$$R = \frac{\sum||F_{CL}| - |F_{NT}||}{\sum|F_{CL}|}$$

where F_{CL}: the structure factor for cross-linked crystal.

F_{NT}: the structure factor for untreated crystal.

nium ion (1,2). The excess concentration of glutaraldehyde is essential in order to achieve partial cross-linking of the protein crystals during the time when rapid reaction is going on between the glutaraldehyde and ammonium sulfate. The partially cross-linked crystals from step 1 are observed to be stable in water for several hours which is long enough for step 2 to complete the reaction without causing disorderness in the crystals. Comparison of the pertinent properties for the cross-linked protein crystal and the untreated one are listed in Table 1.

Our results from the investigation of this particular acid protease provide a sucessful example. One good heavy atom derivative, UO_2^{2+}, was treated in three different ways and developed into three good quality derivatives. The derivatives whose relevant data are listed in Table 1 are prepared as follows:

UA: The cross-linked crystals are stained in $5x10^{-3}$M $UO_2(Ac)_2$ aqueous solution, pH=7, for 48 hours (3 major sites, Table 2 UA).

Table 2 Heavy Atom Parameters

(Results of 3.5A Refinement for Cross-linked Crystals)

		Z † occupancy (% of atom)	x $\times 10^3$	y $\times 10^3$	z $\times 10^3$	B $(T=\exp(-2B\frac{\sin^2\theta}{\lambda^2}))$
UA	1	0.73	447	000	201	0.0
	2	0.90	677	386	121	-19.0
	3	0.60	597	216	258	36.4
			plus 7 minor sites (Z=0.10 - 0.25)			
UB	1	0.85	441	000	201	5.6
	2	0.48	836	009	756	46.8
	3	0.75	595	219	251	20.3
	4	0.62	723	436	724	37.6
	5	0.62	804	393	520	12.3
	6	0.43	680	386	126	-8.7
	7	0.33	644	475	880	11.2
			plus 2 minor sites (Z=0.10 - 0.25)			
UC	1	0.97	438	001	202	8.8
	2	0.47	848	005	759	33.9
	3	0.77	596	220	248	15.0
	4	0.55	722	428	731	52.0
	5	0.88	812	400	522	25.4
	6	0.87	679	389	124	-7.7
			plus 8 minor sites (Z=0.10 - 0.25)			
Pt*	1	0.33	821	386	526	27.7
	2	0.90	300	461	642	4.6
			plus 2 minor sites (Z=0.10 - 0.25)			

† The empirical form factors for the uranyl as well as the Pt derivative, dropped rapidly to ½ its initial value between $(\sin\theta^2/\lambda^2)$=0-0.005 and then flattened out afterwards. If the atomic form factors were used the occupancies should be almost halved.

* Pt extends to 5A only

UB: Untreated crystals are washed in 2.7M $(NH_4)SO_4$ several times, and stored overnight to remove most of the phosphate. It is then stained in saturated $UO_2(Ac)_2$ in 3M $(NH_4)_2SO_4$ for 24 hours (7 major sites, Table 2 UB). It is essentially similar to that found by Jenkins et al (10). The salt concentration is increased from 2.7M to 3.0M in order to modify the cell dimensions.

UC: UB crystals are cross-linked in 8% glutaraldehyde for one hour in 1M $(NH_4)_2SO_4$ while holding $UO_2(Ac)_2$ concentration constant. The crystals are then taken out from the dark slurry mixture and washed in a solution (0.8M $(NH_4)_2SO_4$, 0.1M $UO_2(Ac)_2$). Then they are mounted in capillaries and are ready for use (6 major sites, Table 2 UC).

There are still more derivatives which could be prepared by changing the ionic strength (0-3M $(NH_4)_2SO_4$) and concentration of uranyl (5×10^{-3}-0.1M).

A fourth derivative of K_2PtCl_4 was prepared by soaking cross-linked crystals in 10^{-3}M K_2PtCl_4 aqueous solution, pH 3-6, for 20 hours (2 major sites, Table 2 Pt).

A map (m=0.85) of 3.5A resolution has been completed and the backbone structure of this enzyme, interpreted. The results which provide a comparison with the recently published results on untreated crystal (11) are forth coming.

REFERENCES

1. Quiocho, F.A., & Richards, F.M. (1964) Proc. Nat. Acad. Sci. USA, 52, 833-839.
2. Quiocho, F.A., Bishop, W.H. & Richards, F.M. (1967) Proc. Nat. Acad. Sci. USA, 57, 525-528.
3. Steitz, T.A., Ludwig, M.L., Quiocho, F.A., & Lipscomb, W.N. (1967) J. Biol. Chem., 242, 4662-4668.
4. Sigler, P.M., & Blow, D.M. (1965) J. Mol. Biol., 14, 640-648.
5. Banaszak, L.J., Watson, H.C. & Kendrew, J.C. (1965) J. Mol. Biol. 12, 130-138.
6. Low, B.W., Preston, H.S., Sato, A., Rosen, L.S., Searl, J.E., Rudko, A.D. & Richardson, J.S. (1976) Proc. Nat. Acad. Sci. USA, 73, 2991-2994.
7. Sardinas, US Pat. 3, 275, 453, Sept. 27, 1966.
8. Hagemeyer, K., Fawwal, I. & Whitaker, J.R. (1968) J. Dairy Sci. 51, 833-839.
9. Moews, P.C. & Bunn, C.W. (1970) J. Mol. Biol. 54, 395-397.
10. Jenkins, J.A., Blundell, T.L., Tickle, I.J. & Ungaretti, L. (1976) J. Mol. Biol., 99, 583-590.
11. Subramanian, E., Swan, I.D.A., Liu, M., Davis, D.R., Jenkins, J.A., Tickle, I.J. & Blundell, T.L. (1977) Proc. Nat. Acad. Sci. USA 74, 556-559.