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Structure of Horse Liver Alcohol Dehydrogenase

II. Heavy-atom Derivatives of Type A Crystals

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Three isomorphous heavy-atom derivatives of horse liver alcohol dehydrogenase have been prepared by forming complexes with $K_2Pt(CN)_4$ and $KAu(CN)_2$. The platinum and the gold complexes bind to different characteristic sites, one per subunit. Centric projection data of the orthorhombic crystals were collected to 3 Å resolution for the native enzyme, the two single derivatives, and the double derivative containing both platinum and gold. The heavy-atoms were located from difference Patterson and difference Fourier maps, and the parameters were refined by the method of least-squares. An analysis of the results of this refinement shows that these derivatives give a good phase-determination out to 3 Å resolution. The mean figure of merit varies from 0.97 at low angles to 0.78 at high angles. The Kraut R-factor varies from 0.07 to 0.12. The E-values, which are an estimated error in the structure-factors, are smaller than the heavy-atom contributions for all spacings and all derivatives.

Horse liver alcohol dehydrogenase (LADH) catalyses the oxidation of alcohol to aldehyde when in the presence of the cofactor nicotinamide-adenine-dinucleotide. It is one of the many NAD-dependent dehydrogenases which are composed of two or four subunits, each of molecular weight between 35 000 and 40 000. Of these a low resolution X-ray study has been reported for lactate dehydrogenase [1] and preliminary X-ray data for glyceraldehyde-3-phosphate dehydrogenase,[2] soluble malate dehydrogenase,[3] and liver alcohol dehydrogenase.[4]

We have earlier shown [4,5] that LADH crystallizes with a crystallographic two-fold axis through the dimeric molecule in the absence of coenzyme. This crystal modification, type A, has orthorhombic symmetry, space-group $C222_1$ with cell-dimensions $a = 56$ Å, $b = 75$ Å, and $c = 181$ Å.

The crystallographic molecular symmetry is lost in crystals where coenzyme molecules are bound to the enzyme.

We report here the preparation and investigation of heavy-atom derivatives of type A crystals.

PREPARATION OF CRYSTALS

Purified homogeneous LADH,[6] isoenzyme 3, has been very generously provided to us by Dr. Å. Åkeson at the Nobel Medical Institute, Stockholm. Large crystals were grown as described earlier,[4] except that 2-methyl-2,4-pentanediol was used as precipitant instead of ethanol. Dialysis bags or microdiffusion cells [7] containing suitable crystals of type A were in general dialysed against a solution containing 20 ml 0.05 M tris/HCl, pH 8.4, 17 % v/v of 2-methyl-2,4-pentanediol and 10^{-3} M of the appropriate heavy-metal salt. The enzyme crystals were usually dialysed against this heavy-atom containing solution for a month. Crystals containing $K_2Pt(CN)_4$ and the double derivative $K_2Pt(CN)_4+KAu(CN)_2$ were, however, prepared by cocrystallization of 10^{-5} M enzyme solution and 10^{-3} M $K_2Pt(CN)_4$ or 5×10^{-4} M $K_2Pt(CN)_4+5\times10^{-4}$ M $KAu(CN)_2$, respectively, in 0.05 M tris/HCl, pH 8.4, using 2-methyl-2,4-pentanediol as precipitant.

When we started to try various heavy-metal salts we very soon found that almost any —SH reagent denatured the enzyme crystals. This is not very surprising in view of the fact that the enzyme molecule contains 28 cysteines, two of which are very reactive.[8] We thus had to look for heavy-metal complexes which did not react with —SH groups and which were soluble at pH 8.4. A survey of the literature showed that some of the heavy transition metal cyanide complexes might be suitable. We were fortunate enough to find that $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$ bound to the enzyme at different single sites giving good isomorphous derivatives. It was also possible to prepare the double derivative containing both $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$.

COLLECTION AND MEASUREMENT OF INTENSITIES

Crystals of suitable size ($1.0\times0.3\times0.15$ mm) were mounted at +4°C in the usual way in glass capillaries in equilibrium with their mother liquor. Since these crystals have three centric projections, we decided to do the analysis of our heavy-atom derivatives from high-resolution projection data. 15.5 degrees precession photographs corresponding to a resolution of 3 Å of the *h*0*l*, 0*kl*, and *hk*0 layers were taken at +4°C with Cu$K\alpha$-radiation.

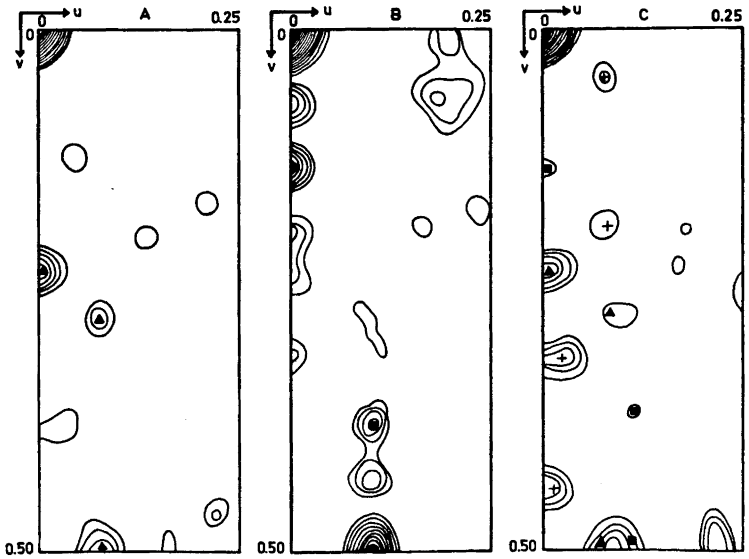

Fig. 1. Difference Patterson projections $P(u,v)$ of heavy-atom derivatives of LADH. (A) $K_2Pt(CN)_4$. (B) $KAu(CN)_2$. (C) $K_2Pt(CN)_4+KAu(CN)_2$.

Approximately 90 % of the possible reflexions could be measured to this resolution. Each crystal lasted for about 25 h irradiation without any measurable damage. Four different exposure times were used for each layer, and the data were recorded on Ilford Industrial G X-ray film.

The intensities were measured as peak intensities on a Joyce-Loebl recording microdensitometer. The symmetry elements of space-group $C222_1$ allow the intensity of each independent reflexion of the centric projections to be determined by four measurements on each film. Approximately 2000 reflexions were measured for each compound.

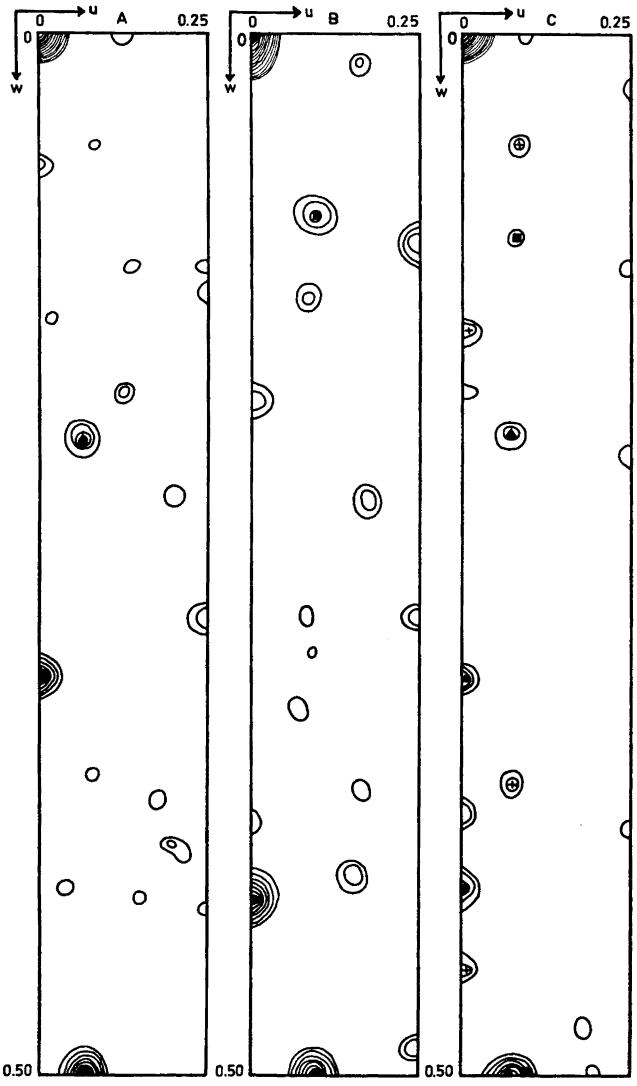

Fig. 2. Difference Patterson projections $P(u,w)$ of heavy-atom derivatives of LADH. (A) $K_2Pt(CN)_4$. (B) $KAu(CN)_2$. (C) $K_2Pt(CN)_4+KAu(CN)_2$.

Lorentz and polarization corrections were applied to the mean value of these four measurements.

Data have been collected for the native enzyme, two single derivatives containing $K_2Pt(CN)_4$ and $KAu(CN)_2$, respectively, and the double derivative from these two heavy metal complexes.

DETERMINATION AND REFINEMENT OF HEAVY-ATOM PARAMETERS

The heavy-atom positions were determined from difference Patterson and difference Fourier maps. The platinum and the gold sites were easily located from difference Patterson projections $P(u,v)$ and $P(u,w)$ of the singly substituted derivatives (Figs. 1 and 2). There was no indication of more than one site per derivative in these maps. The correct relative origin choice was deduced from all three difference Patterson projections of the double derivative $K_2Pt(CN)_4+KAu(CN)_2$, and as a further check Rossmann-type correlation functions [10] of the platinum derivative *versus* the gold derivative were calculated in two projections.

In order to check for minor heavy-atom sites we computed difference Fourier projections of the three derivatives (Figs. 3 – 4) using phase information for each derivative from the remaining two derivatives. No indication of minor sites was found in any derivative. Although extra peaks appear

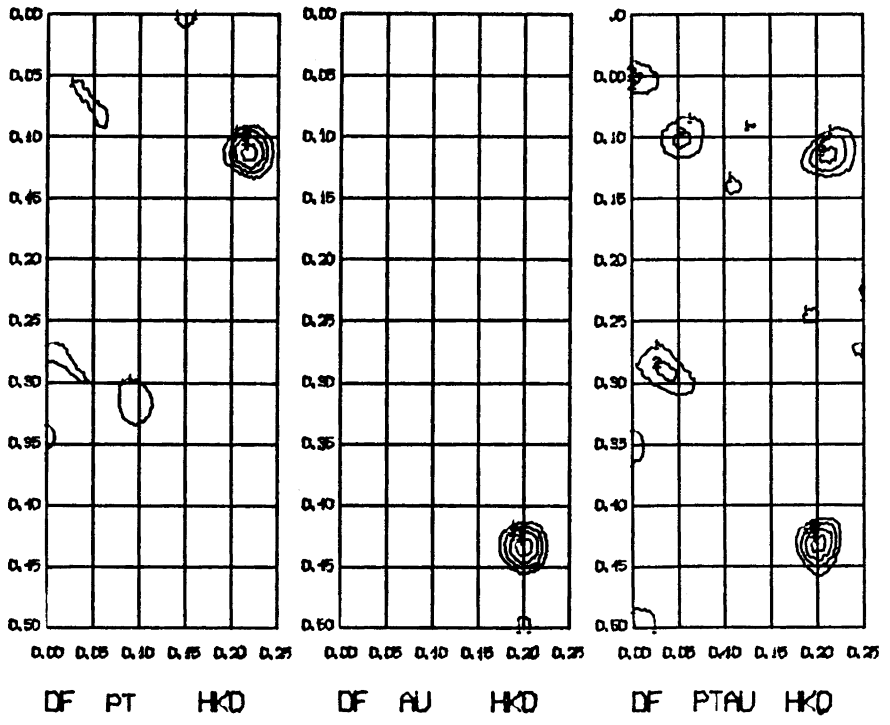

Fig. 3. Difference Fourier projections $\phi(x,y)$ of heavy-atom derivatives of LADH.

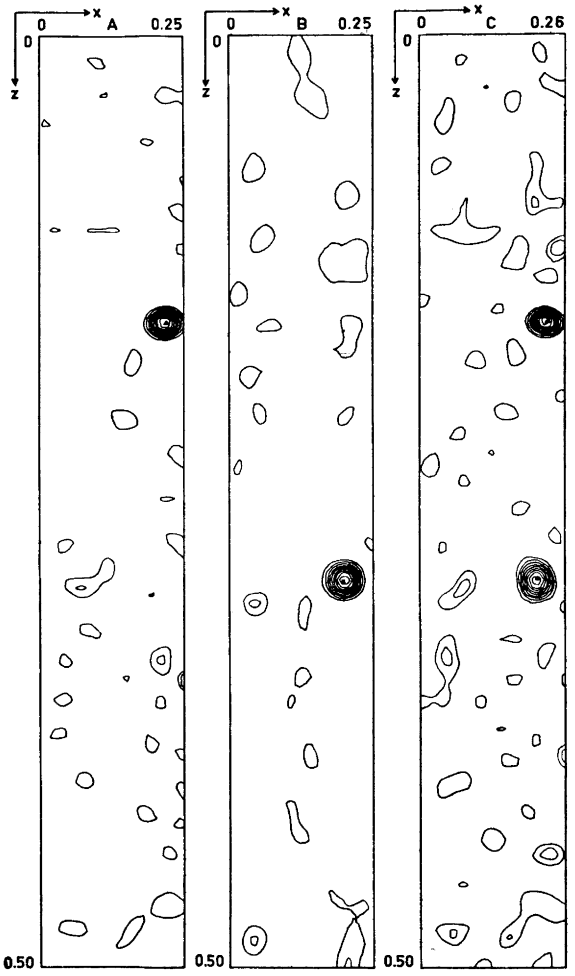

Fig. 4. Difference Fourier projections $\phi(x,z)$ of heavy-atom derivatives of LADH. (A) $K_2Pt(CN)_4$. (B) $KAu(CN)_2$. (C) $K_2Pt(CN)_4+KAu(CN)_2$.

in some *xy*-projections there are no corresponding peaks in the *xz*-projections. Most of the *xy*-maps have shown a fairly high background, and in some cases extra peaks occur probably due to the very long axis of projection, 181 Å, and the fairly small number of independent reflexions in this projection. We cannot exclude, however, that the final three-dimensional analysis will reveal the existence of minor heavy-atom sites.

The heavy-atom parameters has been refined by the method of least-squares using a programme written by one of us, B.-O. S. The main principles of this programme are the same as those described by Dickerson and Weinzierl,[11]

and which have been used in the work on carboxypeptidase [12] and haemoglobin.[13] Alternate cycles of sign determination and refinement of parameters were performed. Merged *hk*0 and *h*0*l* data were used in the refinement.

Positional parameters and occupancies of the heavy-atoms as well as two scale-factors for each derivative, one for each layer, were refined. The isotropic temperature factor B has so far been fixed to a value of 30.0. This value was found from a Wilson-plot of the observed *h*0*l* platinum differences to 2.5 Å resolution. The absolute scale has been adjusted, so that the occupancy of the platinum site is 78 electrons in the singly substituted Pt-derivative. The final parameters are given in Table 1.

Table 1. Final heavy-atom parameters. The B-values have been kept at constant values during the refinement.

Derivative	Heavy atom	Occupancy	x	y	z	B	E_j	R_S	R_K	No. of reflexions
1	Pt	78.0	0.2207	0.1131	0.1538	30	132	0.49	0.09	348
2	Au	73.2	0.2987	0.0631	0.2077	30	105	0.45	0.07	307
3	Pt	45.8	0.2185	0.1147	0.1538	30	122	0.51	0.07	290
	Au	47.8	0.2969	0.0649	0.2083	30				

Criteria which have been used to follow the refinement and to assess the usefulness of the derivatives for phase-determination are summarized in Table 2 as a function of interplanar spacing. The mean figure of merit for the complete set of data varies from 0.97 at low angles to 0.78 at high angles, which is very satisfactory. The overall R-values which are listed in Table 1 are slightly better than those obtained for example on cytochrome *c*.[14] The E-values, which are an estimated error in F_{HP}, are smaller than the observed heavy-atom contribution for all spacings and all three derivatives, which is a strong indication that all derivatives give meaningful phase-information to 3 Å resolution. From the analysis of the results of this refinement we can thus conclude that there is strong hope that these derivatives should give a sufficiently accurate phase-determination to 3.0 Å resolution to produce an interpretable three-dimensional electron density map.

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Table 2. Summary of refinement of 3.0 Å projection data (*h0l* and *hk*0) for three heavy-atom derivatives of LADH.

Derivative		Spacing Å									
		>10.0	10.0–6.7	6.7–5.5	5.5–4.7	4.7–4.2	4.2–3.9	3.9–3.6	3.6–3.3	3.3–3.15	3.15–3.0
1. Pt	E_j	188	128	108	100	120	143	128	129	142	112
	RMSΔF	306	304	279	256	251	252	238	219	196	218
	R_K	0.10	0.08	0.08	0.08	0.07	0.08	0.10	0.12	0.10	0.11
	R_S	0.61	0.43	0.36	0.40	0.47	0.56	0.45	0.59	0.66	0.46
2. Au	E_j	127	90	108	90	119	119	80	92	114	96
	RMSΔF	276	280	265	226	256	232	163	155	170	166
	R_K	0.07	0.06	0.07	0.05	0.06	0.07	0.06	0.06	0.09	0.07
	R_S	0.42	0.34	0.39	0.38	0.46	0.51	0.50	0.59	0.68	0.55
3. Pt+Au	E_j	163	107	137	111	134	122	93	104	94	119
	RMSΔF	276	251	235	246	235	192	201	192	161	203
	R_K	0.09	0.07	0.09	0.07	0.07	0.07	0.07	0.07	0.06	0.08
	R_S	0.56	0.44	0.53	0.44	0.57	0.56	0.42	0.47	0.59	0.54
	$\langle m \rangle$	0.95	0.97	0.93	0.94	0.91	0.90	0.84	0.78	0.86	0.80
	RMSF	1591	1428	1384	1278	1602	1528	1083	1098	1106	1052

F_P = vector scattering from the protein.
F_{HP} = vector scattering from the heavy-atom derivative.
f = heavy-atom vector.
$E_j = [\sum_{hkl}(|F_{HP}|-|F_P+f|)^2/n_j]^{1/2}$
where n_j is the number of observed $|F_{HP}|$ for derivative j.
RMSΔF is the root mean square observed contribution of the heavy atoms for each derivative.
$R_S = \sum_{hkl}|F_{HP}-F_P-f|/\sum_{hkl}||F_{HP}|-|F_P||$
$R_K = \sum_{hkl}||F_{HP}|-|F_P+f||/\sum_{hkl}|F_{HP}|$
$\langle m \rangle$ = mean figure of merit. For centric data

$$m = \frac{P(180)-P(0)}{P(180)+P(0)}$$

where P(0) = probability that the phase angle is 0, and P(180) = probability that the phase angle is 180.
RMSF = root mean square structure amplitude of native protein.

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