Vol. 86, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1979 Pages 1044-1050

A SYSTEMATIC APPROACH TO ISOMORPHOUS REPLACEMENT USING A SERIES OF SIMPLE CHARGED MERCURIALS

Stephen G. Withers, Daniel S.C. Yang, Neil B. Madsen & Robert J. Fletterick

Department of Biochemistry and the Medical Research Council
Group on Protein Structure and Function,
University of Alberta,
Edmonton, Alberta,
Canada T6G 2H7

Received December 5, 1978

SUMMARY. Three novel mercury derivatives bearing different electronic charges have been synthesized and used to prepare useful heavy atom derivatives of crystals of glycogen phosphorylase a. While exhibiting some common binding sites on the exterior of the molecule, their internal binding sites are determined primarily by their charge as predicted. Their ease of preparation and general applicability makes them useful candidates for the preparation of heavy atom derivatives of any protein crystals in X-ray crystallographic studies.

The perennial problem of heavy metal atom substitution into protein crystals as a means of solving the phase problem encountered in X-ray crystallographic investigations has been addressed in many ways. The most commonly used is the trial and error method of addition of various heavy metal salts to the crystal in the hope that they will bind exclusively to one or two sites to give an isomorphous derivative (1). This method, however, often requires many hundreds of crystals and much time to obtain a useful derivative.

The use of more systematic methods of substitution, though desirable, has not generally been too successful, except in some rather specific cases. One of the simpler systematic approaches involves exchange of the metal atom of a metalloprotein with a metal atom of high atomic number, as in the substitution of mercury for zinc in carboxypeptidase (2), while another successful approach involves the preparation of heavy atom labelled specific inhibitors, as with the binding of 5'-iododeoxyuridine 3',5'-

0006-291X/79/041044-07$01.00/0

diphosphate to nuclease (3). The replacement of an amino acid with a heavy atom labelled analogue should be a more general yet systematic approach, but as yet has proved unsuccessful; while the use of protein prosthetic groups as binding sites, as employed with the heme group of myoglobin (4) is not a generally applicable technique. A great need therefore exists for a simple systematic, yet general method for heavy atom labelling. One approach to this is the use of a simple charged heavy metal compound which could only diffuse to a limited number of sites at which covalent binding could take place. Use of similar compounds, but of opposite charge would give different substitution patterns. Such a series of compounds is described in this communication.

MATERIALS AND METHODS

Materials

Crystals of rabbit muscle phosphorylase a were prepared as described previously (5).

Mercuric oxide was obtained from Fischer Chemicals, β-mercaptoethylamine hydrochloride from ICN, thioglycollic acid from Mann Research Laboratories and mercaptoethanol from Sigma Chemical Company.

Synthesis of mercurials

Mercuric oxide (14.3g = 20 m.mol.) suspended in water was added slowly to a solution of the thiol compound (40 m.mol.) in dilute aqueous sodium hydroxide, keeping the solution at pH 8 by addition of sodium hydroxide as necessary. Any excess mercuric oxide was then removed by filtration and the filtrate reduced to a solid or gum on the rotary evaporator. The compounds were crystallised and recrystallised by solution in the minimum volume of the first solvent in the appropriate system shown in Table I, then addition of sufficient of the second solvent to cause some precipitation of material. Scratching the flask aided crystallisation.

Light sensitivity necessitated their storage in the dark, and decomposition on heating precluded melting point determinations. Mercury analyses were performed by volumetric (6) or colorimetric (7) techniques, and are reported in Table I.

Soaking Conditions

Crystals of phosphorylase a were soaked for 24 hours at ambient temperature in the buffer system, 10 mM BES, 50 mM glucose, 10 mM magnesium acetate, 0.1 mM EDTA, pH 6.7 containing the mercury compound (0.4 mM). Diffraction data were collected to a resolution of 4.5 Å using the method-

TABLE I

MERCURY ANALYSES AND CRYSTALLISATION SOLVENT SYSTEMS FOR MERCURIALS

Compound	Theor. % Hg.	Found % Hg.	Solvent System
$Hg(SCH_2COONa)_2$	47.0	46.4	NaOH(0.1N)/Ethanol
$Hg(SCH_2CH_2NH_3Cl)_2$	47.1	47.7	HCl(0.1N)/Ethanol
$Hg(SCH_2CH_2OH)_2$* $\cdot HSCH_2CH_2OH$	46.5	46.3	Methanol/Ether

* This compound appeared to co-crystallise with one mole of mercaptoethanol. This was borne out by elemental analyses ($HgC_6H_6O_3S_3$. Theor. C, 16.7; H, 3.5: Found C, 15.9; H, 3.3) and mass spectral data which indicated the presence of only mercury and mercaptoethanol ligands.

ology previously described (5) with a Syntex $P2_1$ diffractometer, and were analysed by subtraction of the data for the native crystal, followed by a search for density peaks, using the standard difference Fourier technique. Approximately one-half the observable reflections to 4.5 Å resolution (2800 hkl points) were measured for each case.

RESULTS AND DISCUSSION

Under the conditions reported above, tight binding of these compounds to the enzyme would be predicted to occur only at sites bearing a substantial complementary charge or to freely accessible sites of small or zero complementary change which contain a reactive cysteine residue. Covalent binding will be exclusively to sulfhydryl residues since rupture of the relatively stable Hg-S bond necessitates exchange with another bond of similar energy. The sulfhydryl group is the only thermodynamically feasible acceptor. For such an exchange reaction to occur it is also necessary for the compound to diffuse very close to the sulfhydryl, thus the presence in the site of a net charge of the same sign as the compound will preclude tight binding and exchange. The binding pattern of these compounds to phosphorylase a was chosen as a test of their specificity since the structure of this enzyme is now known to a resolution of 2.5 Å (8) so the binding sites could easily be determined by difference Fourier analysis.

TABLE II
MERCURIAL SITE COORDINATES AND OCCUPANCIES ON PHOSPHORYLASE a

Compound	Site	x	y	z	Occ.* %
	EM 2	0.196	0.231	0.038	54
$Hg(SCH_2CH_2OH)_2$	EM 3	0.162	0.381	0.320	47
	EM 1	0.023	0.092	0.385	23
$Hg(SCH_2COO^-)_2$	EM 2	0.196	0.231	0.038	100
	EM 2	0.185	0.219	0.038	93
	DM 2	0.035	0.104	0.333	43
$Hg(SCH_2CH_2NH_3^+)_2$	EM 3	0.162	0.381	0.320	30
	EM 1	0.023	0.092	0.397	25
	DM 1	0.092	0.150	0.282	25

* Occupancies are expressed relative to the largest observed value, that for $Hg(SCH_2COO^-)_2$.

The results of these binding experiments are shown in Table II.

Previous work with phosphorylase b had shown that the most useful mercurial derivative was ethylmercurythiosalicylate (EMTS) which undergoes an exchange reaction with various sulfhydryl groups (9). Soaking crystals with conventional mercurials such as methylmercuric chloride or p-chloromercurybenzoic acid resulted in cracking and extensive disorder. In the case of phosphorylase a, four EMTS sites were found (5) and are referred to below in the order of their occupancy since the compounds described herein occupied these same sites. More recent studies (8) using a mixed mercury reagent (DM) containing dimercuryacetic acid and the positively charged mercurial described here produced new binding sites which are again referred to in the order of their occupancy.

A total of five binding sites is observed with these mercurials. The primary site, which is occupied by all three derivatives at a high level, is the site EMTS 2, on cysteine residue 782 in an alpha helix. This is a very open site with no net associated charge, and is freely accessible to the buffer. Multiple binding at this site is therefore unsurprising. Another external site, EMTS 3, on cysteine residue 171, is also a highly occupied site which is occupied by both the positively charged and the neutral compound, but not the negatively charged compound.

A third site, EMTS 1, which is occupied by the same two mercurials is an internal site on cysteine residue 108. There are a total of 8 acidic groups and only 4 basic groups within a radius of 10 Å, so the predominant charge of this site will be negative. Exclusion of the negatively charged compound is therefore unsurprising.

Probably the most interesting site is that occupied at about 40% exclusively by the positively charged mercurial; the site DM2. This is a totally internal site containing no cysteine residues, but which is surrounded by a cluster of negatively charged acid residues. Within a radius of 10 Å, there are a total of 8 acidic groups and only one basic group. The overall charge of this site must therefore be negative, so the positively charged mercurial is bound sufficiently tightly by electrostatic forces alone to give a good occupancy. This site was used in the phasing for the 2.5 Å data on the enzyme. It is a known metal ion binding site for Gd^{3+}, Yb^{3+}, Mn^{2+} (10,11) again by virtue of the large number of proximal acidic groups. The occurrence of this type of binding further enhances the case for the generality of these reagents, since it is not even necessary for the protein to contain cysteine residues for binding to occur. There is one sulphur containing residue, methionine 99, close to this mercury which may provide some binding assistance of a non-covalent nature. Instances of binding of mercurials to methionine residues are rare, but have been noted, for example in the binding of HgI_4^{2-} to rubredoxin (1).

The least occupied site, DM1, occupied at 20% only by the positively charged mercurial, is again an internal site on cysteine residue 494. This site is also close to the cluster of acidic residues, containing a total of 8 acidic and 4 basic residues, giving the site a predominant negative charge, leading to exclusion of the negatively charged mercurial.

The binding patterns of these compounds are therefore very much as predicted, and these derivatives, particularly the negatively charged, single site mercurial would have proved extremely useful in solving the heavy atom problem of phosphorylase a, an enzyme with nine sulfhydryl groups, by a Patterson map.

Their general applicability and ease of synthesis should lend them directly to use by other crystallographers as useful candidates for heavy metal derivatisation.

ACKNOWLEDGEMENT

This investigation was supported by grants from the Medical Research Council of Canada.

REFERENCES

1. Blundell, T.L. and Johnson, L.N. (1976). Protein Crystallography, pp. 192-235.

2. Tilander, B., Strandberg, B. and Friborg, K. (1965). J. Mol. Biol., 12, 740-760.

3. Arnone, A., Bier, C.J., Cotton, F.A., Day, V.W., Hazen, E.F., Richardson, D.C., Richardson, J.S. and Yonath, A. (1971). J. Biol. Chem. 246, 2302-2316.

4. Bluhm, M.M., Bodo, G., Dintzis, H.M. and Kendrew, J.C. (1958). Proc. Roy. Soc. (London). A246, 369-389.

5. Fletterick, R.J., Sygusch, J., Murray, N., Madsen, N.B. and Johnson, L.N. (1976). J. Mol. Biol. 103, 1-13.

6. Makarova, L.G. and Nesmeyanov, A.N. (1967). Methods of Elemento-Organic Chemistry, Vol. 4, pp. 510-511.

7. Sandell, E.B. (1944). Colorimetric determination of traces of metals. Interscience Pub. Inc. pp. 322-324.

8. Sprang, S. and Fletterick, R.J. Manuscript in preparation.

9. Johnson, L.N., Madsen, N.B., Mosley, J. and Wilson, K.S. (1974). J. Mol. Biol. 90, 703-717.

10. Dwek, R.A., Radda, G.K., Richards, R.F. and Salmon, A.G. (1972). Eur. J. Biochem. 29, 509-514.

11. Fletterick, R.J. Unpublished Results.