Crystal structure study of *Opsanus tau* parvalbumin by multiwavelength anomalous diffraction

R. Kahn*+, R. Fourme*†, R. Bosshard*, M. Chiadmi*, J.L. Risler°, O. Dideberg and J.P. Wery

*LURE, CNRS-Université Paris-Sud, 91405 Orsay Cedex, +Physicochimie Structurale, Université Paris-Val de Marne, 94010 Créteil, †Biologie Physicochimique (GR 13 CNRS), Université Paris-Sud, 91405 Orsay Cedex, °Centre de Génétique Moléculaire, CNRS, 91190 Gif-sur-Yvette, France, and Cristallographie, Institut de Physique-B5, Université de Liège (Sart-Tilman), B 4000 Liège, Belgium

Received 24 September 1984

The crystal structure of a small calcium-binding protein, the parvalbumin III_f from *Opsanus tau* in which Tb was substituted for Ca, has been analysed by multiwavelength anomalous diffraction. Data at a resolution of 2.3 Å were collected at three wavelengths near the L_3 absorption edge of Tb (1.645–1.650 Å), using the synchrotron radiation emitted by a storage ring and a multiwire proportional counter. The phases of the reflections were determined from this single derivative, without native data. Prior to any refinement, the resulting electron density map shows a good agreement with the model of the homologous carp parvalbumin in regions of identical amino-acid sequence.

Multiwavelength anomalous diffraction *Protein crystallography* *Parvalbumin* *Anomalous scattering* *Synchrotron radiation* *Electronic area detector*

1. INTRODUCTION

It is a common practice in protein crystallography to exploit the information derived from the anomalous scattering of heavy atoms, as an aid to help phasing the reflections. In a diffraction experiment at a single wavelength where Bijvoet pairs of reflections are measured, this information is not in itself sufficient to give a unique determination for each phase. Additional information must be provided, for instance from the classical multiple isomorphous replacement (MIR) method, or, more seldom, from the single isomorphous replacement (SIR) technique [1] or from the partial structure of anomalous scatterers (resolved-anomalous phasing) [2]. In contrast with the SIR or MIR methods, multiple wavelength anomalous diffraction (MAD) can provide unambiguous phases [3,4] with only one crystal; this method is thus free of the problems and limitations related to the lack of isomorphism between the native crystal and the heavy-atom derivatives, and eliminates the crucial step of obtaining several independent derivatives. As compared with the resolved-anomalous phasing method, the extended information provided by measurements at multiple wavelengths makes the MAD method more powerful and more general. We report here the determination of the structure of a small protein (parvalbumin) labelled with terbium, using the MAD technique.

The multiwavelength anomalous diffraction (MAD) technique is very difficult to apply with conventional X-ray sources [5]. It is only recently, following the advent of X-ray tunable sources at storage rings combined with films [6] or area detectors [7–10], that a renewed interest in the method arose. Indeed, tunability is essential to take full advantage of the anomalous dispersion, since data can be recorded at various wavelengths in the immediate vicinity of an elemental absorption edge. Of special interest are those elements which exhibit threshold resonances, the so-called 'white lines', in their X-ray absorption spectrum, because of their particularly large anomalous scattering factors [11]. In the range of experimentally accessible

Published by Elsevier Science Publishers B.V.
00145793/85/$3.30

wavelengths, the best candidates are the L_3 edges of elements lying between Cs and Pt [12], including the lanthanides, which are of common use as heavy atom labels in macromolecules.

Parvalbumin III_f of the superfast swimbladder muscle of *Opsanus tau* was chosen for several reasons, in addition to its intrinsic interest as a calcium-binding protein: it is a small protein (molecular mass 10098 Da); it binds two Ca^{2+} which can be substituted by lanthanides, a well established method to study the structure and function of Ca-binding proteins [13]; and finally, one could expect its 3-dimensional structure to be similar to the refined carp parvalbumin structure [14,15].

The results described here have been presented at a satellite meeting of the eighth International Biophysics Congress [16].

2. MATERIALS AND METHODS

O. tau parvalbumin was prepared as described in [18]. Tb binding to the protein in solution was checked by PIXE (proton induced X-ray emission) [19] and fluorescence [20] and crystals of the Tb-labelled parvalbumin were grown in ammonium sulphate (to be published). The crystal data are: space group $P2_12_12$, a = 56.1 Å, b = 59.4 Å, c = 27.8 Å, Z = 4 molecules/unit cell. The crystals are rectangular parallelepipeds (dimensions ~0.15 × 0.9 × 0.15 mm^3), elongated along the b axis. Diffraction extends to better than 1.7 Å on photographs taken with a focused synchrotron radiation (SR) beam.

Diffraction experiments were performed at LURE using the bending magnet X-ray beam line D1 on the positron storage ring DCI. A non-focusing 'channel-cut' monochromator using the Ge(220) reflection for wavelength selection was placed 22 m from the positron orbit. The diffractometer consisted of a single axis goniometer with a spherical drift multiwire proportional chamber [10]. A charge-integrating ionization chamber placed between the collimator and the crystal was used to put the intensity measurements on a common scale. Data collection and preliminary data reduction were performed on-line with a dedicated PDP 11/34 minicomputer. Data sets at a resolution of 2.3 Å were measured at three wavelengths close to the L_3 edge of Tb (~1.650 Å) chosen in order to minimise the average error on phases [21]. The three wavelengths were: (1) at the white line absorption maximum λ_1 = 1.649 Å; (2) at a lower wavelength λ_2 such that $\lambda_1 - \lambda_2$ = 0.0021 Å; (3) at the absorption edge inflection point λ_3 such that $\lambda_3 - \lambda_1$ = 0.0011 Å. The crystal was rotated around the b-axis using steps of 0.002° and diffraction patterns integrated over 0.05° were stored onto disk after data reduction. The wavelength was kept constant through 15° sectors, with each sector measured at the three wavelengths successively. All three data sets were collected within 36 h on the same crystal. The absorption spectrum of a $TbCl_3$ powder sample in the Tb L_3 edge region was recorded at the beginning of each sector. The point of maximum absorption was found to be stable to ± 0.5 eV; this demonstrates the high orientational stability of the SR beam which, together with a long beam lifetime (40 h), were key factors in the success of the experiment. Count rate did not exeed 30000 cps for storage ring conditions of 250 mA and 1.72 GeV circulating positrons energy.

Since the crystal was rotated around the b-axis, Bijvoet pairs (hkl, $h\bar{k}l$) were measured at the same time and with essentially identical absorption effects. Individual reflections were not corrected for absorption. The λ_2-set (for which anomalous effects of Tb are minimal) was put on an absolute scale using intensity statistics. The same scale factor was applied to the λ_3-set (f_3' close to f_2'), but the λ_1-set which is the most affected by absorption and has f_1' close to f_2', was rescaled to have the same average intensity as the λ_2-set.

3. RESULTS AND DISCUSSION

The average individual standard deviation of the integrated intensities was 3–4% as estimated from the counting statistics, and the average $\sigma(I)/I$ after merging of intensities was 7%. This shows that the electronic area detector meets the requirements for high precision data recording.

The Tb partial structure was solved by anomalous Patterson techniques [22] using the λ_1-set and it was refined against the 96 strongest anomalous differences to an R-index of 0.24, using the program ANOLSQ [2]. Two sites were found. The first one has a high occupancy (≃1) and turns out to correspond to the CD site for Ca^{2+} in carp

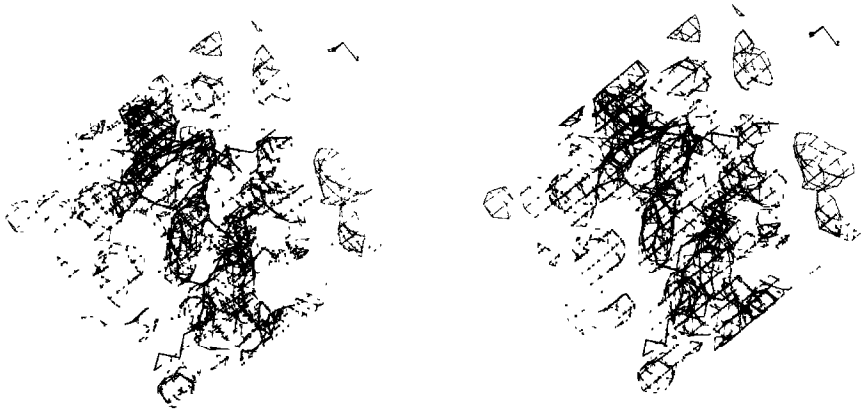

Fig.1. Stereo representation of the fragment Leu65–Ala72 from the carp parvalbumin model [12] superimposed onto the 2.3 Å resolution MAD electron-density map of *O. tau* parvalbumin. Atomic coordinates for the model were derived by molecular replacement (see text), without any further modification. Both amino acid sequences are identical in this region of the chain, which is part of an α-helix.

parvalbumin. The second one corresponds to the EF site and has a low occupancy ($\simeq$0.3). In initial stages of this work [17] this second site was not taken into account.

For general (acentric) reflections, the phasing technique was derived from MIR methods [23], as used by others to exploit anomalous scattering effects at one [2] or several [24] wavelengths. In contrast with the MIR case, (MAD) experiments are free from isomorphism errors: all measurements are made on the same crystal and all of the data are of similar quality. The total phase probability distribution $P(\phi)$ can thus be taken as the product of those distributions formed from all possible pairings of one particular data set (called here the reference set) with the other sets [24]. In the present study, six sets of measurements were recorded, one set of (*hkl*) and one set of ($h\bar{k}l$) for each of the three wavelengths. It was thus possible to calculate five 'lack of closure' values $\epsilon(\phi)$ for each reflection of the reference set (*hkl* at λ_1). For the constrained-phase reflections, an original method was devised which treats the various data sets on an equal basis and considers as random variables the amplitudes as well as the phases of the wavelength-independent part of the (*hkl*) structure factor. A detailed account of this phasing method, which has now been extended to acentric reflections, will be published elsewhere.

White line features depend on the environment and bonding of the anomalous scatterer [11] and on the monochromator bandpass. It is thus essential to derive accurate values for f_n' and f_n'' from the diffraction measurements themselves. A Bijvoet pair of reflections (10 5 1 and 10 $\bar{5}$ 1) was chosen for their large variation in intensity when scanning the wavelength λ across the Tb L_3 edge. Since the difference between the intensities of such reflections (corrected for absorption effects) is proportional to f'', the variation of f'' as a function of λ in the region of interest can be determined. The correlated variation of f' was obtained by the Kramers-Kronig dispersion relation [25]. The initial values of all f_n' and f_n'' were refined by minimization of $\Sigma\epsilon^2(\phi)$ over all reflections for suitable pairings. The centroid phases, the F_i' and the $\Delta F_{ij}'$ were refined in a cyclic procedure and led to the following values: $f_1' = -14.1$, $f_2' = -12.4$, $f_3' = -28.0$, $f_1'' = 19.9$, $f_2'' = 12.0$, $f_3'' = 10.2$*

MAD electron density maps based on 2439 phased reflections (figure of merit 0.73) were calculated for the two enantiomorphs. One of these maps showed an unambiguous chain tracing.

*f_n', f_n'': real and imaginary corrections to the scattering factor of the anomalous scatterers at wavelength n; F_i' (F_i''): amplitude of the real (imaginary) component of anomalous scatterers structure factors in data set i where $i = +n\,(-n)$, the index of the *hkl* ($h\bar{k}l$) set of reflections at wavelength n; $\Delta F_{ij}'' = F_i'' - F_j''$

Recently two of us (J.P.W. and O.D.) have independently solved the Tb-parvalbumin structure by the molecular replacement technique [26], using 3.2 Å data measured with CuK_α radiation and the known carp parvalbumin molecule as a model. The carp parvalbumin structure could thus be superimposed onto our MAD map, and proved to fit nicely the electron density in regions of identical amino acid sequences as shown in fig.1. The average phase discrepancy for the 721 reflections which are common to both determinations is 54°, prior to any refinement of either structure. This demonstrates the basic correctness of the two independent structure determinations of *O. tau* parvalbumin, and proves that multiwavelength anomalous diffraction is now a feasible and mature technique. Note also that a preliminary application of multiwavelength measurements using an algebraic method for phase determination has been recently reported for lamprey hemoglobin [27]. The refinement of the structure using the *O. tau* amino acid sequence is under way.

ACKNOWLEDGEMENTS

The area detector is the result of a long-term collaboration with G. Charpak's group at CERN, Geneva. W.A. Hendrickson and J.L. Smith kindly sent us programs for anomalous resolved phase determination. Discussions with J.C. Phillips on MAD methods were quite stimulating. C. Gerday purified the parvalbumin and checked the binding of terbium by spectroscopic methods. G. Weber performed the PIXE analysis. S. Brunie, J. Janin, A. Rimsky and M. Chiadmi contributed to various stages of this work. Finally, we thank P. Marin and the technical staff of the LAL who operated the storage ring DCI.

REFERENCES

[1] Blow, D.M. and Rossmann, M.G. (1961) Acta Crystallogr. 14, 1195–1202.
[2] Hendrickson, W.A. and Teeter, M.M. (1981) Nature 290, 107–113.
[3] Raman, S. (1959) Proc. Ind. Acad. Sci. 50A, 95–107.
[4] Herzenberg, A. and Lau, H.S.M. (1967) Acta Crystallogr. 22, 24–28.
[5] Hoppe, W. and Jakubowski, U. (1974) in: Anomalous Scattering (Ramaseshan, S. and Abrahams, S.C. eds) pp.437–461, Munksgaard, Copenhagen.
[6] Phillips, J.C., Wlodawer, A., Goodfellow, J.M., Watenpaugh, K.D., Sieker, L.C., Jensen, L.H. and Hodgson, K.O. (1977) Acta Crystallogr. A33, 445–455.
[7] Arndt, U.W. (1978) Nucl. Instr. Methods 152, 307–311.
[8] Phizackerley, R.P., Cork, C.W., Hamlin, R.C., Nielsen, C.P., Vernon, W., Xuong, N.H. and Perez Mendez, V. (1980) Nucl. Instr. Methods 172, 393–395.
[9] Mokulskaya, T.D., Kuzev, S.V., Lubnin, M.Y., Myshko, G.E., Mokulskii, M.A., Smetanina, E.P., Baru, S.E., Kulipanov, G.N., Sidorov, V.A. and Khapakhpashev, A.G. (1980) Abstr. 6th European Crystallographic Meeting, Barcelona.
[10] Kahn, R., Fourme, R., Bosshard, R., Caudron, B., Santiard, J.C. and Charpak, G. (1982) Nucl. Instr. Methods 201, 203–208.
[11] Phillips, J.C., Templeton, D.H., Templeton, L.K. and Hodgson, K.O. (1978) Science 201, 257–259.
[12] Wendin, G. (1980) Phys. Scripta 21, 535–542.
[13] Reuben, J. (1975) Naturwissenschaften 62, 172–178.
[14] Moews, P.C. and Kretsinger, R.H. (1975) J. Mol. Biol. 91, 201–228.
[15] Moews, P.C. and Kretsinger, R.H. (1975) J. Mol. Biol. 91, 229–232.
[16] Kahn, R., Fourme, R., Bosshard, R., Chiadmi, M., Risler, J.L., Brunie, S., Dideberg, O., Wery, J.P. and Janin, J. (1984) Communication at the Symposium on New Methods in X-Ray Absorption, Scattering and Diffraction for Application in Structural Biology, Bristol, England.
[17] Kahn, R., Fourme, R., Bosshard, R. and Charlier, P. (1982) Abstr. 7th European Crystallographic Meeting, Jerusalem.
[18] Hamoir, G., Dideberg, O. and Charlier, P. (1981) J. Mol. Biol. 91, 201–228.
[19] Johansson, T.B., Akelsson, R. and Johansson, S.A.E. (1970) Nucl. Instr. Methods 84, 141–143.
[20] Sowadski, J., Cornick, G. and Kretsinger, R.H. (1978) J. Mol. Biol. 124, 123–132.
[21] Narayan, R. and Ramaseshan, S. (1981) Acta Crystallogr. A37, 636–641.
[22] Rossmann, M.G. (1961) Acta Crystallogr. 14, 383–388.
[23] Blow, D.M. and Crick, F.H.C. (1959) Acta Crystallogr. 12, 794–802.
[24] Phillips, J.C. and Hodgson, K.O. (1980) Acta Crystallogr. A36, 856–864.

[25] James, R.W. (1980) in: The Optical Principles of the Diffraction of X-Rays, Cornell University Press, Ithaca.
[26] Rossmann, M.G. and Blow, D.M. (1962) Acta Crystallogr. 15, 24–31.
[27] Hendrickson, W.A. (1984) Communication at the Symposium on New Methods in X-Ray Absorption, Scattering and Diffraction for Application in Structural Biology, Bristol, England.