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Selenomethionine Incorporation in *Saccharomyces cerevisiae* RNA Polymerase II

Technical Advance

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Summary

A protocol for the incorporation of SeMet into yeast proteins is described. Incorporation at a level of about 50% suffices for the location of Se sites in an anomalous difference Fourier map of the 0.5 MDa yeast RNA polymerase II. This shows the utility of the approach as an aid in the model-building of large protein complexes.

Introduction

Selenomethionine (SeMet) incorporation has become a standard technique in protein crystallography. It is useful both for phase determination by multiwavelength anomalous dispersion (MAD) and for the location of methionine residues as an aid to polypeptide chain tracing. In most cases, SeMet incorporation has been done in *Escherichia coli*, where complete replacement of methionine with SeMet is routinely accomplished [1]. Many eukaryotic proteins, however, cannot be expressed in *E. coli* because they are too toxic, require posttranslational modification, or are made up of many subunits, whose coexpression is problematic. These proteins may be produced with the use of baculovirus vectors in insect cells [2] or, in the case of multisubunit complexes, by isolation from yeast or higher cells without the benefit of overexpression. Protocols for SeMet incorporation in the baculovirus system have recently been described, but incorporation was limited by the ability of the insect cells to grow on SeMet-containing media [3–5]. We have encountered similar difficulties in the incorporation of SeMet into RNA polymerase II (pol II) from the yeast *Saccharomyces cerevisiae*. This 10 subunit, 0.5 MDa protein had been purified in a form suitable for crystallization from a strain of yeast in which the gene for the fourth largest subunit, Rpb4, was deleted. An electron density map that revealed the course of the polypeptide chain was obtained by multiple isomorphous replacement with anomalous scattering (MIRAS) [6]. SeMet markers would be of particular value to confirm the backbone model and to guide the placement of the amino acid sequence in such a large protein complex. We were unable to grow a yeast methionine auxotroph on SeMet and achieve complete incorporation. However, we were prompted to pursue the partial incorporation of SeMet by previous evidence that the lower anomalous signal from incomplete incorporation could be detected. This evidence came from soaking pol II crystals in Mn^{2+} solution. A single peak with a height of 6 σ was observed in a Mn anomalous difference Fourier map computed with the final MIRAS phase set despite the comparatively small Mn anomalous effect (two electrons at the wavelength used). Since Se shows an anomalous signal of 4 electrons at the peak energy, we could hope to detect Se sites with only about 50% SeMet substitution. While the Se signal might have limited phasing power, the Se sites would still be of great value as markers for chain tracing. SeMet markers were obtained as described here and proved to be of particular value to confirm the backbone model and to guide the placement of the amino acid sequences in pol II (P. C., unpublished data).

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Results and Discussion

Initial experiments employed a yeast methionine auxotroph, with SeMet as the sole source of methionine. Cells were grown on synthetic complete medium to an A_{600} of 1.0, after which they were transfered to medium containing SeMet instead of methionine. Cell growth ceased in the presence of SeMet, and the incorporation of SeMet into total proteins reached about 30% (Table 1). To improve the incorporation, we sought to sustain cell growth by the use of media containing methionine as well as SeMet. We also investigated the use of a nonauxotroph since SeMet inhibits the normal methionine biosynthesis pathways and reduces the availability of methionine [7]. The auxotroph proved capable of growth on SeMet and methionine in a ratio of 9 to 1, while the nonauxotroph grew on SeMet alone. Maximal incorporation of 65% SeMet into total proteins of the nonauxotroph was attained (Table 2). These growth conditions were used for preparation of SeMet-containing pol II.

Pol II was prepared from a nonauxotrophic strain bearing a deletion of the gene for the fourth largest subunit, Rpb4, shown previously to be important for crystallization. Upon introduction into a fermentor, growth of this strain normally lags for about 12 hr and then proceeds with a doubling time of about 4.5 hr. In the presence of SeMet, the lag was about 48 hr, and the doubling time was about 15 hr. Pol II that was purified from cells grown under these conditions contained 55% SeMet. Crystallization was as described [6, 8] except that the SeMet protein required a slightly lower concentration of PEG 6K in the precipitant solution. The crystals took about twice as long to grow (2–4 weeks), were smaller, and did not diffract to as high resolution as did native crystals of a similar size. A scan of X-ray–induced fluorescence from a small SeMet crystal (0.1 mm $\times$ 0.1 mm $\times$ 0.05 mm) showed a Se absorption peak at 0.979 Å (12,662 eV). Data were collected at this wavelength, processed with Denzo, and reduced with SCALEPACK [9]. The processing statistics were as follows: mosacity, 0.77 degrees; linear R_{sym} (50–3.8 Å), 11%; linear R_{sym} (highest resolution shell), 23%; multiplicity, 8.1; completeness, 99.0%; $R_{anomalous}$ (50–3.8), 3.3%. SIRAS phase information extended to about 6 Å (Table 3) and was of sufficient

Table 1. Growth of Yeast Methionine Auxotroph in the Presence of SeMet

Minutes after SeMet addition	0	30	60	90	120	150	180
A_{600}	1.0	1.2	1.4	1.5	1.6	1.6	1.6
Percent SeMet incorporation in total cellular protein (to nearest 5%), strain DY3441	0	<5	10	20	30	30	30
Percent SeMet incorporation in total cellular protein (to nearest 5%), strain DY805	0	<5	10	20	30	30	30

Table 2. Incorporation of SeMet in Yeast Grown on Both Methionine and SeMet

Strain	Media	SeMet (mg/ml)	Methionine (mg/ml)	Growth	A_{600} Final	% SeMet
CB010Δrpb4	SC	0	0.1	Yes	3.1	0
CB010Δrpb4	SC	0.04	0	Yes	2.2	30
CB010Δrpb4	SC	0.1	0	Yes	1.9	65
CB010Δrpb4	SC	0.1	0.01	Yes	2.0	55
CB010Δrpb4	SC	0.1	0.04	Yes	2.5	30
CB010Δrpb4	SC	0.2	0.02	No	ND	ND
CB010Δrpb4	YPD	0	0	Yes	4.4	0
CB010Δrpb4	YPD	0.02	0	Yes	3.3	10
CB010Δrpb4	YPD	0.2	0	No	ND	ND
DY344Δrpb4	SC	0.0	0.1	Yes	3.0	0
DY344Δrpb4	SC	0.04	0	No	ND	ND
DY344Δrpb4	SC	0.1	0	No	ND	ND
DY344Δrpb4	SC	0.1	0.01	Yes	1.2	30
DY344Δrpb4	SC	0.1	0.04	Yes	2.1	10
DY344Δrpb4	SC	0.2	0.02	No	ND	ND
DY344Δrpb4	YPD	0	0	Yes	4.2	0
DY344Δrpb4	YPD	0.02	0	Yes	3.3	10
DY344Δrpb4	YPD	0.2	0	No	ND	ND

ND: not determined.
YPD: 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose.
SC: modified synthetic complete medium.

Table 3. SIRAS Phasing Statistics

Dmin (Å)	Dmax (Å)	$FOM_{acentrics}$	$FOM_{centrics}$	Phasing Power Acentrics (ano/sio)
49.22	10.53	0.772	0.609	2.21/1.71
10.53	7.53	0.679	0.609	1.72/1.70
7.53	6.17	0.497	0.447	1.34/1.40
6.17	5.36	0.236	0.137	1.03/1.21
5.36	4.80	0.220	0.182	0.81/1.06
4.80	4.38	0.151	0.067	0.67/0.84
4.38	4.06	0.113	0.064	0.57/0.49
4.06	3.80	0.098	0.061	0.47/0.44

FOM = figure of merit.
Phasing power: mean value of heavy-atom structure factor amplitudes divided by the lack of closure. Statistics were calculated with Sharp [13].

Table 4. Peaks in 25–4 Å Anomalous Difference Fourier Map

σ Level	SeMet Peaks	Noise Peaks in Protein Mask	Noise Peaks in Solvent	Other Peaks
7+	4	0	0	8 (Zn)
6–7	13	0	0	0
5–6	26	0	0	0
4–5	34	1	2	2 (cysteine)
3.3–4	16	45	38	1 (cysteine)

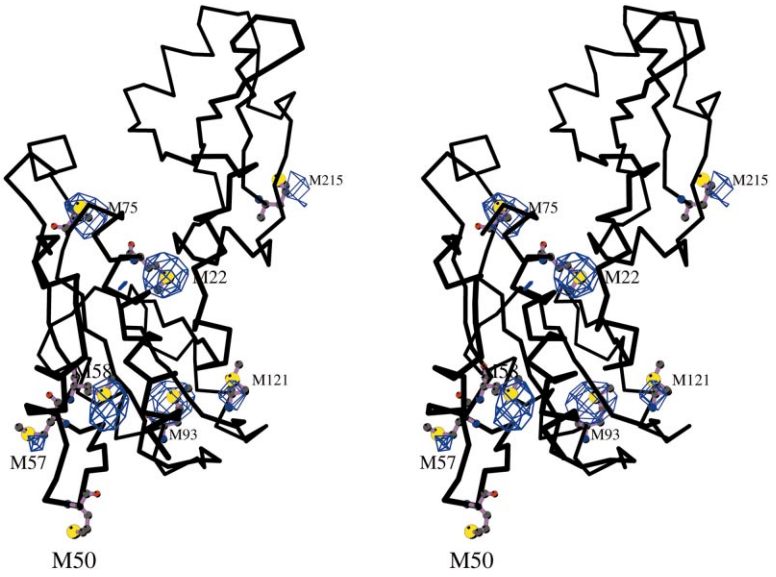

Figure 1. Stereoview of a C_α Trace of Rpb5, with a Se Anomalous Difference Fourier Map Superimposed

The C_α backbone of Rpb5, as determined for the isolated subunit and placed in the experimental density map of RNA polymerase II, is shown in black, with methionine residues highlighted in a ball-and-stick representation (magenta). The Se anomalous difference Fourier map calculated from 25–4 Å and contoured at 3 σ is shown in blue. Peak heights are as follows: Met-22, 6.9 σ; Met-50, not observed; Met-57, 3.7 σ; Met-58, 6.8 σ; Met-75, 5.9 σ; Met-93, 4.3 σ; Met-121, 3.6 σ; Met-215, 3.3 σ. Two noise peaks near the 3 σ cutoff can also be seen.

quality to obtain a low-resolution envelope. A full MAD dataset would presumably have given better results. The data were useful, however, for locating Se sites. An anomalous difference Fourier map computed from 25 to 4 Å with the previously determined MIRAS phase set clearly showed 90 peaks above 4 σ (Table 4). The eight highest peaks corresponded to the Zn sites previously located in pol II. The highest Zn and Se peaks were 15.7 σ and 7.8 σ, respectively. The amino acid sequence of the 10 subunit pol II studied here contains 119 methionine residues, not including those at subunit N termini. The validity of the Se peaks was established by comparison with the known positions of methionine residues in the structure of the Rpb5 subunit, previously determined by X-ray analysis of the isolated subunit [10], and placed in the 3.5 Å polymerase II map [6]. Peaks above 4 σ were observed for all methionine residues in the interior of rpb5, and peaks above 3.3 σ were seen for all but one methionine residue at the surface (Figure 1). The refined pol II structure comprises 103 ordered methionines (P. C., unpublished data). Out of these 103 methionines, 77 had peaks higher than 4 σ in the 25–4 Å anomalous Fourier map (Table 4). There was no strong correlation between the degree of solvent exposure of a methionine residue and its peak height in the anomalous Fourier map. In addition to peaks due to Zn and methionine, three peaks above 3.3 σ corresponded to cysteine residues, including a 3.6 σ peak observed for Cys-207 in subunit Rpb3. The occurrence of some selenocysteine is not surprising since methionine can be used by yeast to produce cysteine [11].

Experimental Procedures

Cell Growth

For initial trials, methionine auxotrophic strains of *Saccharomyces cerevisiae* DY3441 (ade2 can1 his3 leu2 met14 ura3) and DY805 (trp1 ura3 leu2 his3 met2) were grown on synthetic complete medium (0.03 mg/ml each of adenine sulfate, uracil, L-tryptophan, L-histidine–HCl, L-arginine–HCl, L-tyrosine, L-leucine, L-isoleucine, and L-lysine–HCl, 0.05 mg/ml L-phenylanine, 0.1 mg/ml L-glutamic acid, 0.1 mg/ml L-aspartic acid, 0.15 mg/ml L-valine, 0.2 mg/ml L-threonine, 0.4 mg/ml L-serine, 0.1 mg/ml L-cysteine, 0.1 mg/ml L-glutamine, 0.1 mg/ml L-proline, 0.1 mg/ml L-alanine, 0.67% w/v yeast nitrogen base without amino acids (DiFco), and 2% w/v dextrose) containing 0.1 mg/ml methionine to an OD600 of 1.0 absorbance units. Cells were centrifuged aseptically, washed three times with sterile water, and resuspended in synthetic complete medium containing 0.1 mg/ml SeMet (Fluka). Samples of the cell culture were taken at 30 min intervals, centrifuged, washed three times with water, resuspended in lysis buffer (50 mM Tris-Cl [pH 7.5], 100 mM KCl, 1 mM EDTA, 10 mM DTT, and 10% glycerol), and frozen in liquid nitrogen. After they thawed, we lysed the cells by shaking them with glass beads. Samples of whole cell extract were precipitated with trichloroacetic acid and submitted for amino acid analysis (Table 1).

For trials with strains CB010 Δrpb4 and DY3441 Δrpb4, yeast were grown in 50 ml of YPD (1% yeast extract [Vinequary], 2% peptone [US Biological], 2% dextrose) or in 50 ml of modified synthetic complete medium (0.09 mg/ml each of adenine sulfate, uracil, L-tryptophan, L-histidine–HCl, L-arginine–HCl, L-tyrosine, L-leucine, L-isoleucine, and L-lysine–HCl, 0.15 mg/ml L-phenylanine, 0.3 mg/ml L-glutamic acid, 0.3 L-aspartic acid, 0.45 mg/ml L-valine, 0.6 mg/ml L-threonine, 1.2 mg/ml L-serine, 0.34 mg/ml thiamine, 0.12 mg/ml L-cysteine, 0.3 mg/ml L-glutamine, 0.3 mg/ml succinic acid, 0.2 mg/ml L-proline, 0.2 mg/ml L-alanine, 0.01 mg/ml inositol, 1.34% w/v yeast nitrogen base without amino acids (DiFco), and 3% w/v dextrose), with methionine and SeMet as indicated (Table 2). Cell growth was monitored, and when it slowed, the cells were harvested, washed 3 times with water, resuspended in 1 ml of 25 mM Tris-Cl (pH 7.5), 10% glycerol, 100 mM KCl, 1 mM EDTA, and 10 mM DTT, shaken with glass beads for 20 min, and centrifuged. A sample of the supernatant (100 μg protein) was submitted for amino acid analysis (Table 2). Using amino acid analysis, we determined the percentage of SeMet incorporation by monitoring the loss of methionine. Amino acid analysis was performed by AAA Service Laboratory of Boring, OR and the PAN facility at Stanford University.

RNA Polymerase II Purification and Crystallization

Yeast strain CB010 Δrpb4 (100 l) was grown in a Chemap fermentor on modified synthetic complete medium containing 0.1 mg/ml SeMet. The dissolved oxygen content was maintained at 100% air saturation. Cells were grown to an A_{600} of 2.6. This yielded 945 g of wet pellet. RNA polymerase II was purified as described [12]. Using amino acid analysis, we monitored the loss of methionine to determine the percentage of SeMet incorporation. Amino acid analysis of purified polymerase II was performed by AAA Service Laboratory of Boring, OR. SeMet RNA polymerase II crystals were grown as previously described [6, 8]. Data were collected at the Stanford Synchrotron Radiation Laboratory, beamline 9–2. In order to ensure high redundancy and data quality, we took 0.5 degree oscillations with inverse beam geometry by using 60 degree wedges.

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