Supporting Information

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SI Text

Chemical Synthesis of the Alpha-Peptide. The synthesis of the 23mer alpha-peptide (*Ec*POX$_{550-572}$, H-Met-Leu-Arg-Ala-Ile-Ile-Ser-Gly-Arg-Gly-Asp-Glu-Val-Ile-Glu-Leu-Ala-Lys-Thr-Asn-Trp-Leu-Arg-OH) was carried out using an ABI 443A peptide synthesizer running standard Fmoc-chemistry in a 0.125-mmol scale. The attachment of the first amino acid to 2-chlorotrityl-chlorid resin was carried out using an established protocol [Barlos K, *et al.* (1989) Synthesis of Protected Peptide-Fragments Using Substituted Triphenylmethyl Resins. *Tetrahed Lett* 30:3943–3946]. For the following double coupling steps 4 eq amino acid, 4 eq HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluoro-phosphate), 4 eq HOBt (4-hydroxybenzotriazole) and 8 eq DIPEA (*N,N*-diisoproethyl-amine) in DMF (*N,N*-dimethylformamide) were used. The *N*-terminal Fmoc-protection group was cleaved with a solution of 2% piperidine/2% DBU (1,8-Diaza-bicyclo[5.4.0]-7-undecene) in DMF. After complete synthesis the simultaneous cleavage of the peptide from the resin and side chain deprotection were done by a mixture of 5% TIS (triisopropylsilane), 5% water and 90% TFA (trifluoroacetic acid) for 3 h at room temperature. After evaporation of the solvent the peptide was precipated by ice-cold diethylether. The crude product was washed with diethylether, filtered and purified via preparative HPLC using a C18 column employing a water/acetonitrile gradient (with 0.1% TFA) from 5–35% over 80 min. Correct synthesis was confirmed by mass spectrometry. The total yield of the pure product was 16 mg.

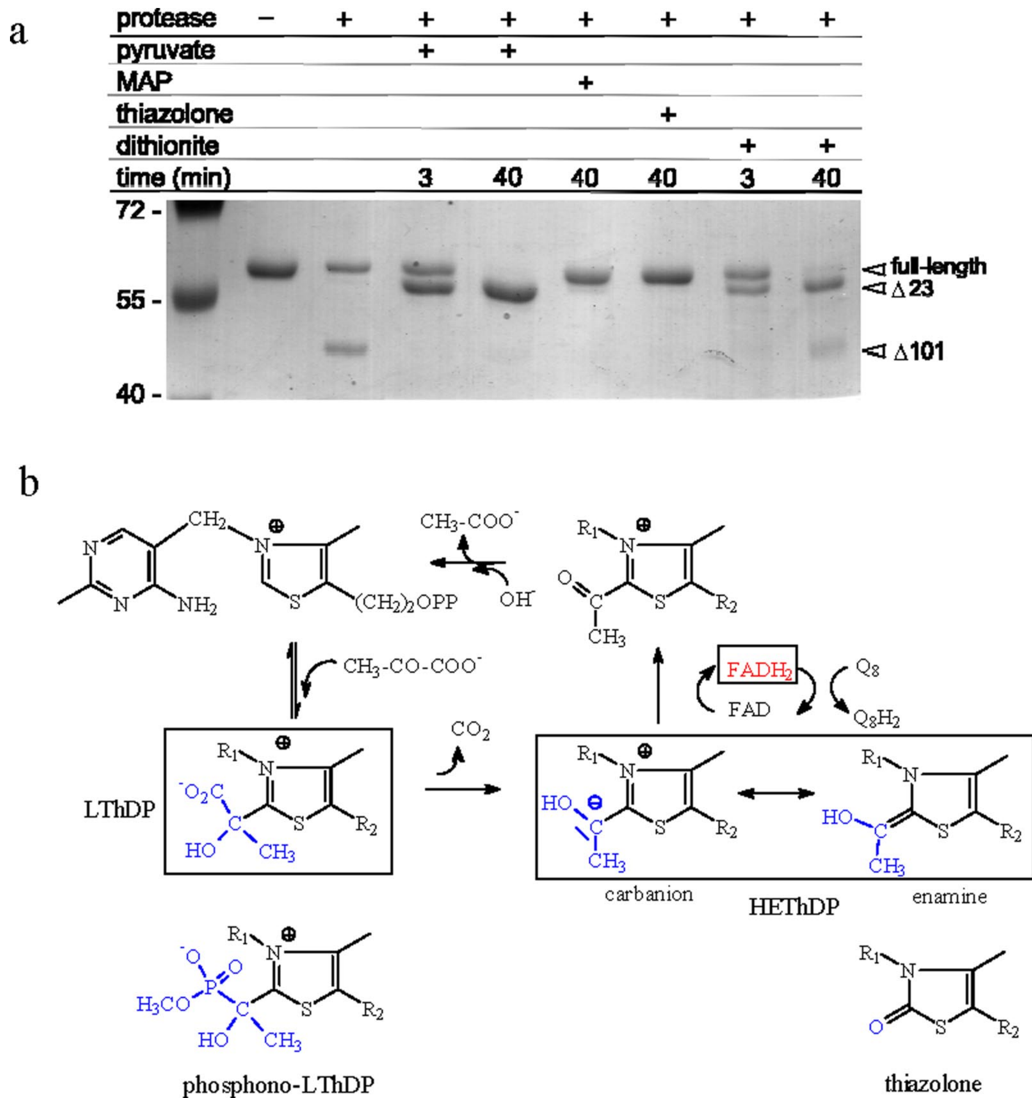


Fig. S1. (*a*) SDS-page analysis of *Ec*POX after treatment with alpha-chymotrypsin at different catalytic stages. Conditions for limited proteolysis were as detailed by Hager and coworkers [Recny MA, Hager LP (1983) Isolation and characterization of the protease-activated form of pyruvate oxidase. Evidence for a conformational change in the environment of the flavin prosthetic group. *J Biol Chem* 258: 5189–5195]. *Ec*POX was subjected to proteolytic digestion (*i*) in the resting state, (*ii*) after reaction with 200 mM pyruvate, (*iii*) after reaction with 20 mM methylacetyl-phosphonate (MAP, a substrate analog) resulting in the formation of the stable predecarboxylation intermediate analog phosphono-LThDP, (*iv*) after reconstitution with 100 μM thiamin thiazolone diphosphate (an analog of the HEThDP enamine), and (*v*) after complete reduction with sodium dithionite under strict anaerobic conditions. (*b*) Catalytic cycle of *Ec*POX with native intermediates and corresponding intermediate analogs indicated.

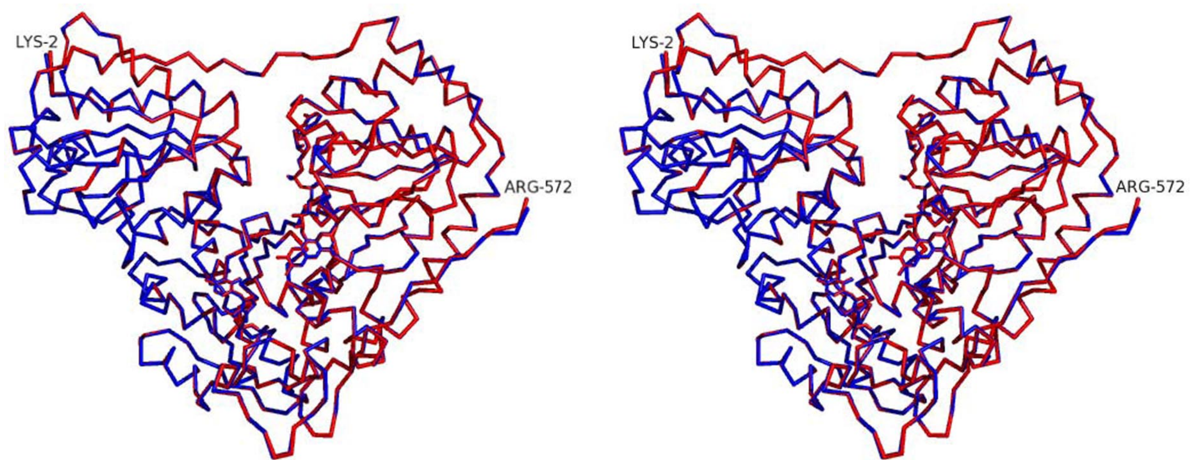


Fig. S2. Superposition of both subunits in the asymmetric unit of full-length *Ec*POX in stereoview and ribbon representation. Chains A and B are shown in blue and red, respectively. The cofactors ThDP and FAD are depicted in stick representation.

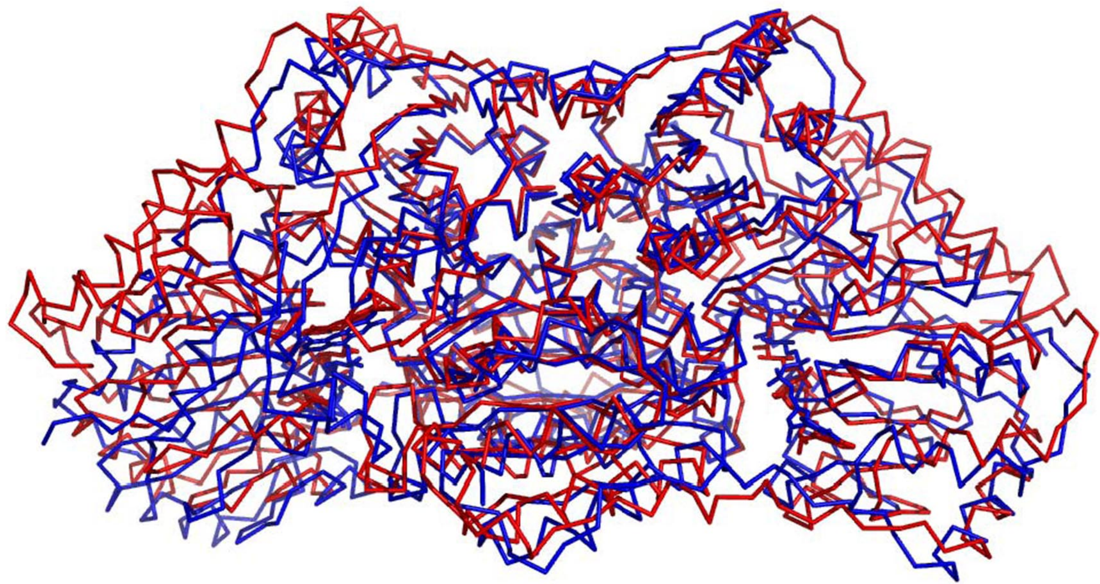


Fig. S3. Superposition of the catalytic dimers of *Lp*POX (red) and *Ec*POX (blue) reveals a similar overall fold of both proteins.

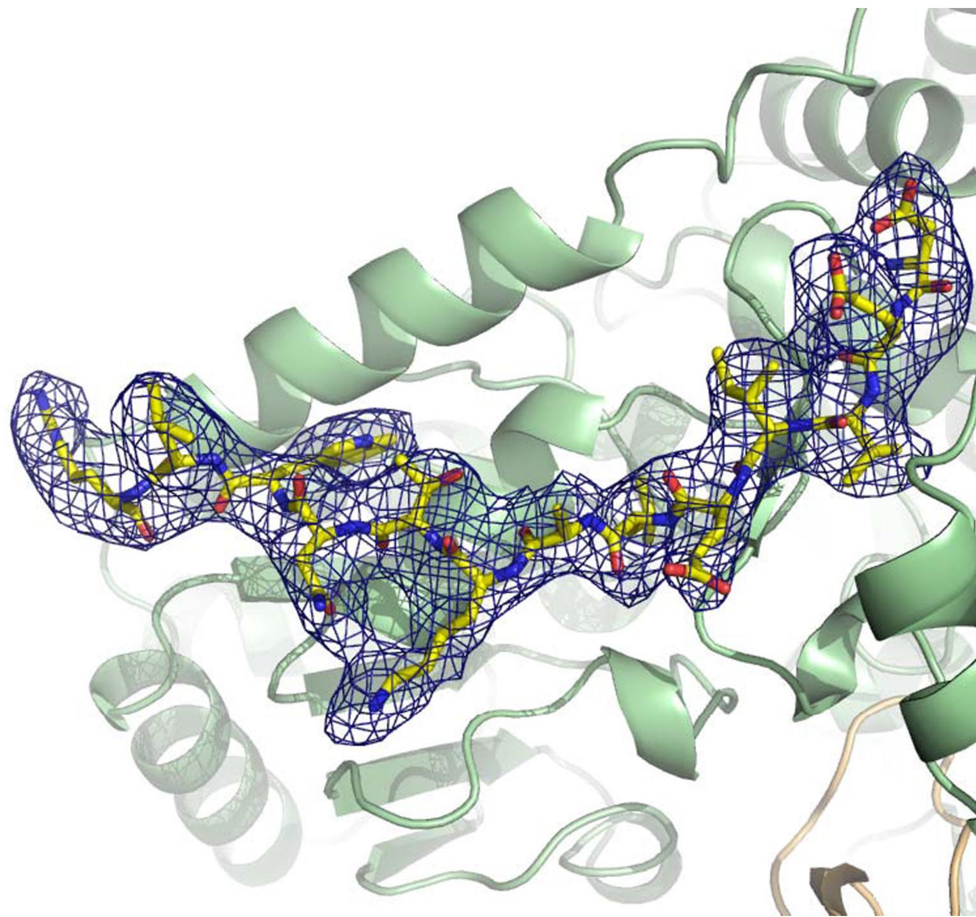


Fig. S4. Structure of the C terminus of full-length *Ec*POX in diagram representation displaying electron density for the last C-terminal 13 residues (shown as sticks) at a contour level of 3σ in a F_o-F_c omit map.

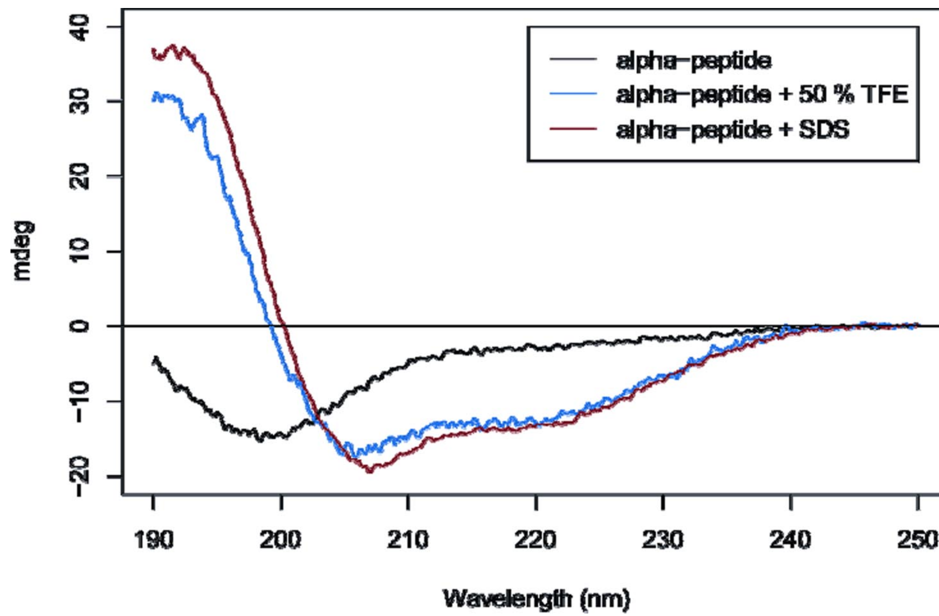


Fig. S5. Far-UV CD spectra of 50 μM alpha-peptide (*Ec*$POX_{550-572}$) in 20 mM potassium phosphate buffer pH 6.0 without further additives (black line), and in presence of either 100 mM SDS (brown line) or 50% trifluorethanol (blue line). The spectra shown were recorded at 20°C and an optical path length of 10 mm at a Jasco J-810 CD spectrometer.

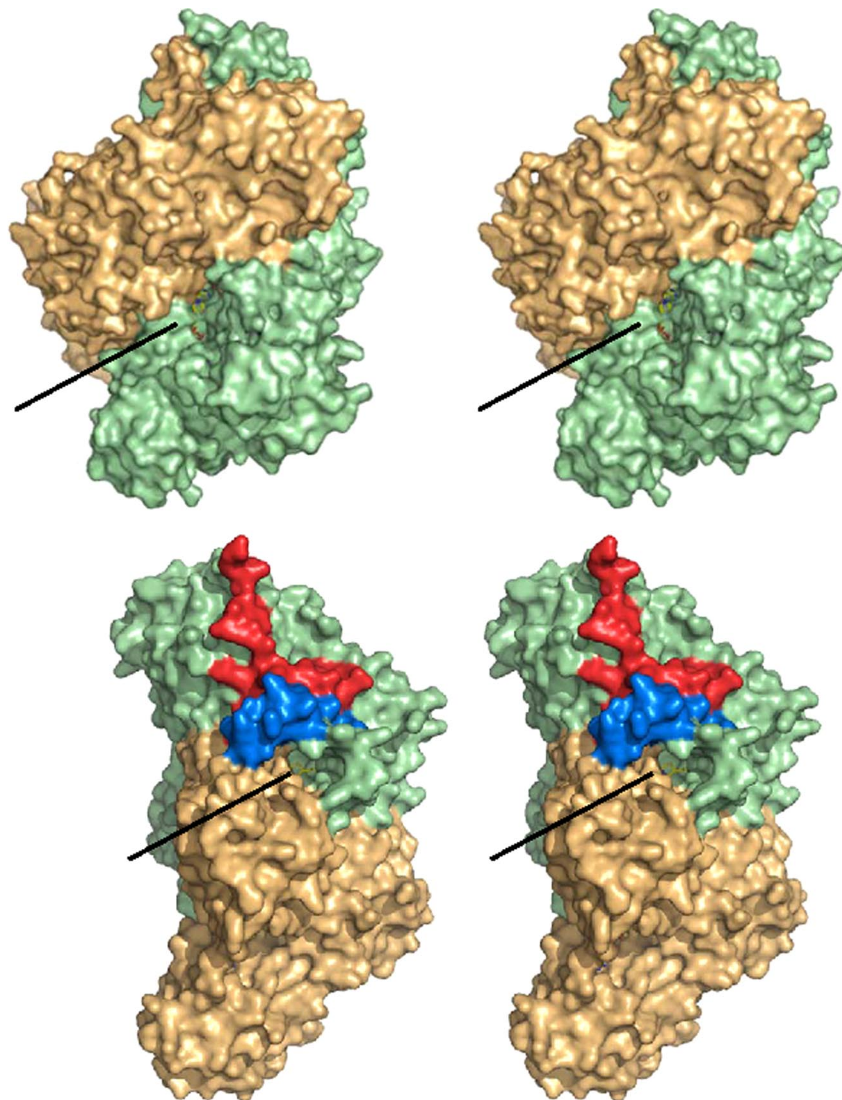
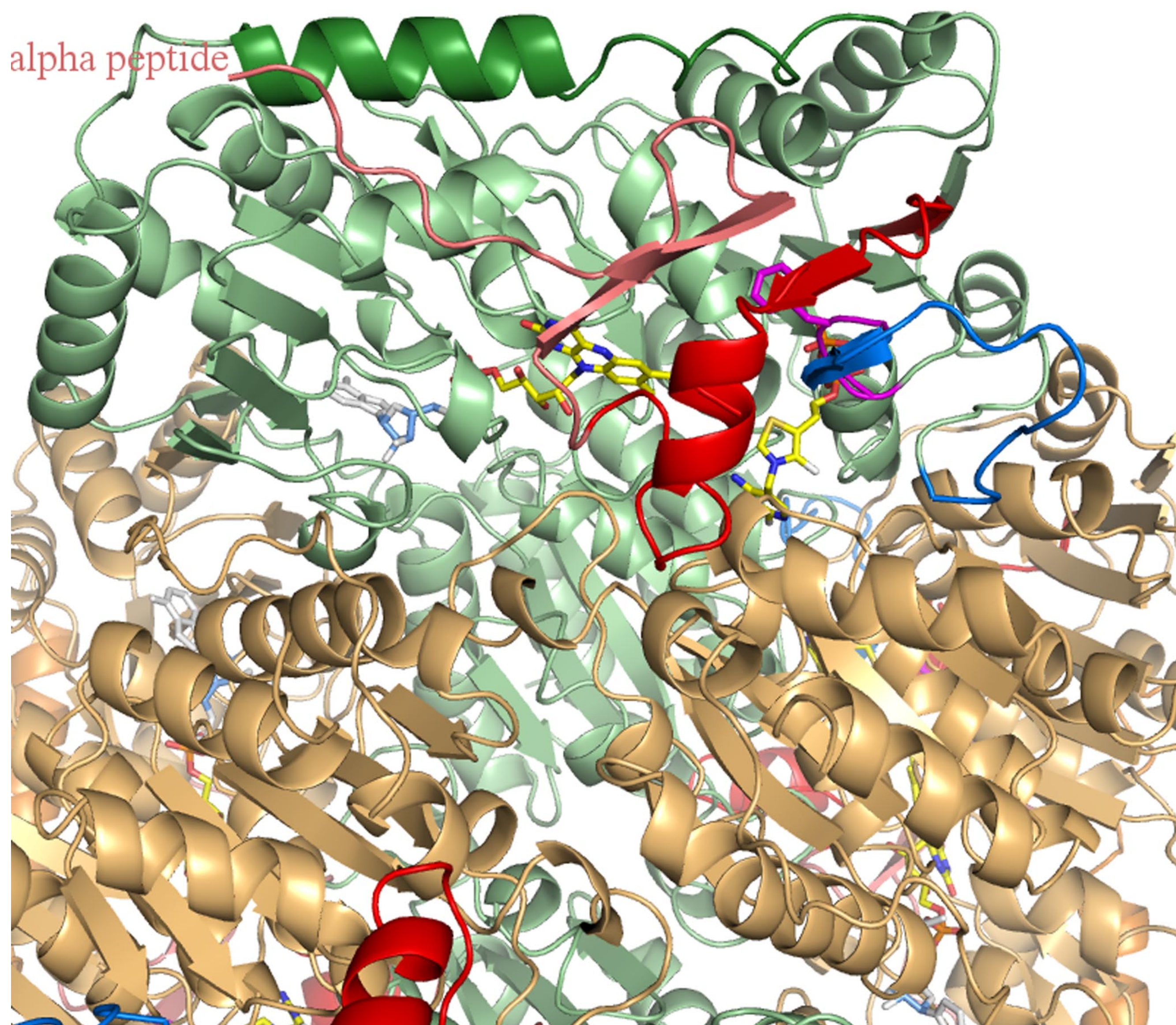


Fig. S6. Surface representation of pyruvate dehydrogenase E1 component from *E. coli* (*Top*) [Arjunan P, *et al.* (2002) Structure of the pyruvate dehydrogenase multienzyme complex E1 component from *Escherichia coli* at 1.85 A resolution. *Biochemistry* 41: 5213–5221] and *Ec*POX (*Bottom*) viewed down the substrate channel. The corresponding subunits of the dimers are coloured individually and the entrance to the active site at the dimer interface is indicated. For *Ec*POX, the linker region (blue) and the AP (red) of the C terminus are highlighted. In both enzymes, the active sites are located at the bottom of a long funnel-shaped substrate channel.

FULL-LENGTH EcPOX



Movie S1. Structural transition of *Ec*POX in the course of activation.

Movie S1 (MOV)

Table S1. Crystallographic statistics

	Full-length EcPOX (1–572)	$EcPOX_{\Delta 23}$ (1–549)
Data collection		
Wavelength, Å	1.54180	0.91840
Space group, Å	P4(3)2(1)2	P2(1)2(1)2(1)
a	151.36	203.24
b	151.36	207.05
c	153.74	214.54
Monomers in asymmetric unit	2	12
Max. resolution, Å	2.9	2.5
R_{merge}, %	7.3 (74.4)	13.1 (70.8)
Completeness, %	98.6 (99.6)	99.7 (98.5)
No. of reflections	417728	1610583
No. of unique reflections	39657	309571
$\langle I/\sigma(I) \rangle$	25.07 (2.54)	20.75 (1.95)
Refinement		
Resolution range, Å	30.0–2.9	30.0–2.5
Completeness (working + test), %	98.79 (99.6)	99.62 (98.21)
No. of reflections ($F > 0$)	39635	294151
Wilson B, $Å^2$	72.85	60.20
R_{cryst}, %	18.32 (32.53)	18.34 (27.50)
R_{free}, %	21.60 (37.90)	19.77 (29.20)
No. of nonhydrogen atoms		
Protein	8,684	47,891
Water	0	1329
Sulfate/Phosphate	10	180
FAD, ThDP, Mg^{2+}	160	960
R.m.SD from ideality		
Bond lengths, Å	0.007	0.013
Bond angles, °	1.129	1.467
Dihedrals, °	19.350	22.895
Improper, °	0.073	0.095
Average B-factor, $Å^2$		
Protein atoms	81.00	60.23
Water		55.34
Sulfate/Phosphate	84.21	73.96
FAD, ThDP, Mg^{2+}	65.11	51.00

$R_{merge} = \Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle$. Values in parentheses correspond to the highest resolution shell. $R_{cryst} = \Sigma ||F_{obs}| - |F_{calc}|| / \Sigma |F_{obs}|$. R_{free} is calculated as for R_{cryst} but for a test set comprising reflections not used in the refinement (5.0%).