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Crystallization Experiments with 2-Enoyl-CoA Hydratase, Using an Automated 'Fast-Screening' Crystallization Protocol

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Abstract

A convenient method for screening crystallization conditions using an automated fast-screen protocol has been implemented and tested on an enoyl-CoA hydratase. The crystallization solutions for the initial screening and subsequent optimizations are prepared using a crystallization robot. Enoyl-CoA hydratase (E.C. 4.2.1.17), purified from rat-liver mitochondria, is one of the enzymes from the β-oxidation pathway of fatty-acid metabolism; it catalyzes the reversible hydration of 2-*trans*-enoyl-CoA's to L-3-hydroxyacyl-CoA's. Different crystal forms, diffracting to 3.0 Å, were obtained.

Introduction

The incomplete-factorial method is a powerful tool to reduce the number of crystallization trials required to identify the influence of different variables in crystallization experiments (Carter & Carter, 1979). Conditions are chosen by systematic assignment of variables, according to the principles of randomization and balance. Variables tested in the screening, such as pH, temperature, precipitant and additive, are based on known properties of the protein and information from crystallization conditions of related proteins (Abergel, Moulard, Moreau, Loret, Cambillau & Fontecilla-Camps, 1991). Care should be taken to avoid conditions favoring the growth of inorganic crystals. A set of 50 screening conditions for the initial screen has been developed (Jancarik & Kim, 1991). Various salt and precipitant compositions with five different buffers are tested in the sparse matrix. Because the same conditions are used in each initial screening this approach is well suited for automation. Crystallization robots have been developed to set up crystallization experiments (Cox & Weber, 1987; Oldfield, Ceska & Brady, 1991). Here we describe the use of the system (Fig. 1), as described by Oldfield, Ceska & Brady (1991), for setting up a crystallization experiment according to the sparse-matrix method. The software provides the necessary instructions for dispensing the stock solutions in the wells. This software is flexible and can easily be used to carry out systematic variations of the crystallization variables.

2-Enoyl-CoA hydratase catalyzes the reversible stereospecific hydration either of $\Delta^{2,3}$-*trans*-enoyl-CoA substrates to the corresponding L-(+)-3-hydroxyacyl-CoA products or of $\Delta^{2,3}$-*cis*-enoyl-CoA's into D-(−)-3-hydroxyacyl-CoA's (Fig. 2) (Waterson & Hill, 1972). In view of the present knowledge, only the reaction mentioned first is considered to participate in the β-oxidation of fatty acyl-CoA in both mammals and bacteria (Hiltunen, Filppula, Häyrinen, Koivuranta & Hakkola, 1993). The rat mitochondrial enzyme has a molecular

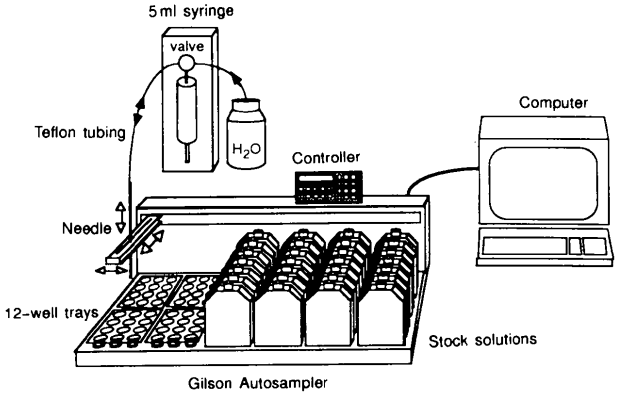

Fig. 1. The crystallization robot. The movement of the needle is indicated by open arrows, the liquid flow by closed arrows.

Table 1. *Composition of the 48 well solutions of the intial screen*

Tray	Buffer* 100 m*M*	(pH)†	Salt‡	Precipitant 1	Concentration	Precipitant 2	Concentration	Crystals§
1A1	—	(8.2)	—	$(NH_4)_3PO_4$	1.5 *M*	—		
1A2	MES	(6.5)	$MgSO_4$	$(NH_4)_2SO_4$	1.5 *M*	—		
1A3	Citrate	(3.1)	NaCl	$(NH_4)_2SO_4$	2.0 *M*	—		
1A4	CHES	(9.5)	—	$(NH_4)_2SO_4$	2.0 *M*	MPD	5%	
1A5	HEPES	(7.5)	—	$(NH_4)_2SO_4$	2.0 *M*	PEG 400	2%	
1A6	Acetate	(4.8)	Li_2SO_4	$(NH_4)_2SO_4$	2.5 *M*	—		
1B1	MES	(6.5)	—	$(NH_4)_2SO_4$	2.5 *M*	Ethanol	10%	277 K
1B2	Tris	(8.5)	—	$(NH_4)_2SO_4$	3.0 *M*	—		
1B3	HEPES	(7.5)	—	*t*-Butanol	30%	—		293 K
1B4	MES	(6.5)	$CaCl_2$	Ethanol	20%	—		277 K
1B5	Citrate	(3.1)	—	Ethanol	30%	—		
1B6	Acetate	(4.8)	—	Ethanol	30%	PEG 6 K	10%	277 K
1C1	Tris	(8.5)	$MgCl_2$	Ethanol	40%	—		
1C2	HEPES	(7.5)	—	Isopropanol	10%	PEG 400	20%	
1C3	Acetate	(4.8)	—	Isopropanol	20%	—		
1C4	Citrate	(3.1)	—	Isopropanol	20%	PEG 8 K	2%	
1C5	CHES	(9.5)	$MgCl_2$	Isopropanol	30%	—		
1C6	—	(5.5)	—	$KNaHPO_4$	2.0 *M*	—		
1D1	HEPES	(7.5)	—	KNa tartrate	1.5 *M*	—		
1D2	Acetate	(4.8)	—	Li_2SO_4	1.0 *M*	—		
1D3	Tris	(8.5)	—	Li_2SO_4	1.0 *M*	$(NH_4)_2SO_4$	1.0 *M*	
1D4	HEPES	(7.5)	—	Li_2SO_4	1.5 *M*	—		
1D5	Citrate	(3.1)	—	$MgSO_4$	1.5 *M*	—		
1D6	Tris	(8.5)	—	$MgSO_4$	2.0 *M*	—		
2A1	CHES	(9.5)	$MgCl_2$	MPD	20%	—		
2A2	Citrate	(3.1)	—	MPD	30%	—		
2A3	Tris	(8.5)	—	MPD	30%	*t*-Butanol	2.5%	293 K
2A4	Acetate	(4.8)	—	MPD	40%	—		
2A5	HEPES	(7.5)	NaCl	MPD	40%	—		293 K
2A6	HEPES	(7.5)	—	Na_3 citrate	1.4 *M*	—		
2B1	CHES	(9.5)	—	NaCl	1.4 *M*	—		
2B2	Tris	(8.5)	—	NaCl	2.0 *M*	—		
2B3	HEPES	(7.5)	—	NaCl	2.0 *M*	PEG 400	10%	
2B4	Acetate	(4.8)	—	NaCl	3.0 *M*	—		
2B5	Acetate	(4.8)	—	Na formate	2.0 *M*	—		
2B6	Citrate	(3.1)	$CaCl_2$	PEG 400	20%	—		
2C1	HEPES	(7.5)	$MgCl_2$	PEG 400	30%	—		
2C2	Tris	(8.5)	—	PEG 400	40%	—		
2C3	Acetate	(4.8)	—	PEG 6 K	20%	NaCl	1.0 *M*	
2C4	Citrate	(3.1)	—	PEG 6 K	20%	*t*-Butanol	2.5%	
2C5	HEPES	(7.5)	—	PEG 6 K	25%	—		
2C6	MES	(6.5)	Li_2SO_4	PEG 6 K	25%	—		
2D1	Acetate	(4.8)	NaK tartrate	PEG 6 K	25%	—		
2D2	Tris	(8.5)	—	PEG 8 K	10%	MPD	5%	293 K
2D3	Citrate	(3.1)	—	PEG 8 K	18%	—		
2D4	MES	(6.5)	$CaCl_2$	PEG 8 K	18%	—		
2D5	CHES	(9.5)	$MgCl_2$	PEG 8 K	20%	—		
2D6	HEPES	(7.5)	—	PEG 8 K	25%	—		

* Each well also contains 1 m*M* DTT, 1 m*M* NaN_3 and 1 m*M* EDTA.

† The number is the pH of the original buffer, as specified in Table 2. It should be noted that the actual pH in the well solution can be significantly different due to the various additions, or the change in temperature.

‡ The salt concentration is 200 m*M*, except for $CaCl_2$ which is 20 m*M*.

§ Crystalline enoyl-CoA hydratase was observed in the indicated wells at the specified temperature.

weight of 161000 Da and consists of six identical polypeptide chains (Furuta, Miyazawa, Osumi, Hashimoto & Ui, 1980). This enzyme shows the highest catalytic rate with C_4 substrates, with decreasing efficiency from C_4-CoA to C_{16}-CoA. The primary structure of the mitochondrial 2-enoyl-CoA hydratase shows significant similarity with mitochondrial $\Delta^{3,2}$-enoyl-CoA isomerase and with the amino-terminal sequence of the peroxisomal multifunctional enzyme from rat (Palosaari & Hiltunen, 1990). 2-Enoyl-CoA hydratase catalyzes the same type of reaction as enolase, the crystal structure of which is known (Stec & Lebioda, 1990), and fumarase. Moderate homology was found between residues 104–144 of 2-enoyl-CoA hydratase and 230–271 of pig-heart fumarase (Minami-ishii, Taketani, Osumi & Hashimoto, 1989). Crystals of pig-heart fumarase (Sacchettini, Meininger, Roderick & Banaszak, 1986) and fumarase from *Saccharomyces cerevisiae* (Keruchenko, Keruchenko, Gladilin, Zaitsev & Chirgadze, 1992) have been described.

Materials and methods

For the crystallization of 2-enoyl-CoA hydratase we used an automated fast-screen protocol. This fast screen was based on a sparse-matrix procedure published previously (Jancarik & Kim, 1991), on crystallization conditions documented in the crystallization data bank (Gilliland, 1988) and using the program *Cristal* (Roussell, Serre, Frey & Fontecilla-Camps, 1990). The conditions were optimized to prevent the formation of inorganic crystals. To simplify interpretation of the results we sorted the crystallization conditions based on the precipitant used (Table 1). For preparation of the 48 well solutions a crystallization robot was used (Oldfield, Ceska & Brady, 1991). The equipment consisted of a standard Gilson Autosampler (x–y–z translator) and a motor-driven syringe (Fig. 1). Four trays (each with 12 wells) or two trays (with 24 wells) and 26 stock solutions, in 50 ml tissue-culture flasks (Falcon) were placed in the Autosampler. The stock solutions, consisting of different buffers, precipitants and additives, were prepared as indicated in Table 2. The stock solutions were transferred to the wells under computer control, using an upgraded version of existing software (Oldfield, Ceska & Brady, 1991). The total volume in each well was 1 ml. The flasks were covered with a tissue to prevent transfer of drops, which sometimes stick to the outside of the needle. The 5 ml syringe and the 5 ml Teflon tubing were filled with water. Up to 5 ml of the first stock solution was aspirated through the needle into the Teflon tubing (a small air bubble was present between the water and the stock solution to prevent mixing). Subsequently, the stock solution was dispensed into the wells according to the input specifications. The injection speed for viscous solutions, such as PEG and MPD was 0.72 ml min^{-1} and for other solutions it was up to 24.0 ml min^{-1}. On completion of the delivery of the first stock solution, the tubing was rinsed with water and the next stock solution was transferred. Preparing the 48 wells took 90 min. Finally, the well solutions were mixed manually. The pH of the well solution is determined by the buffer but will also depend on the presence of the added precipitant, additive and temperature. Crystallization experiments were carried out in 12-well PVC trays (Nelipak, Venray, The Netherlands). The protein drops were produced on 22 mm round glass siliconized cover-slips (1% Prosil-28, PCR incorporated, Gainesville, Florida) by mixing 1 µl protein solution and 1 µl well solution. The protein drops were prepared manually, to minimize evaporation. Two sets of these 48 conditions were prepared: one set was placed at 277 K and one set at room temperature; therefore, 96 conditions were screened. The crystallization conditions were examined immediately and then every day for the first week. If crystals were found, the same 26 stock solutions were used to optimize conditions using the crystallization robot. In the second screening the importance of precipitant concentration, the presence and absence of the additive, and the pH of the solution was tested, by varying one of the parameters in a grid search consisting of three rows and six columns. The precipitant concentration was varied in the first and second row, with and without the additive. Solution pH varied in the third row.

If no crystals were obtained, the fast screen was repeated with the following changes; in all the conditions where the protein precipitated immediately after mixing, the precipitant concentration was halved and for the conditions without visible precipitate, the protein concentration was doubled.

2-Enoyl-CoA hydratase was purified from rat livers. Chromatography with phosphocellulose (P-11) and hydroxyapatite columns and ammonium sulfate precipitation were carried out as described by Furuta, Miyazawa, Osumi, Hashimoto & Ui (1980). The protein from the second precipitation was dissolved in buffer *A* [20 m*M* potassium phosphate, pH

Table 2. *The 26 stock solutions for the automated fast screen*

1.	Water	
2. 50%	MPD	
3. 50%(*v*/*v*)	PEG 400	
4. 40%(*w*/*v*)	PEG 6000	
5. 30%(*w*/*v*)	PEG 8000	
6. 1.0 *M*	Citric acid/NaOH	pH = 3.1
7. 1.0 *M*	Na acetate/HAc	pH = 4.8
8. 1.0 *M*	MES/NaOH	pH = 6.5
9. 1.0 *M*	HEPES/NaOH	pH = 7.5
10. 1.0 *M*	Tris/HCl	pH = 8.5
11. 1.0 *M*	CHES/NaOH	pH = 9.5
12. 50 m*M*	DTT, EDTA, NaN_3	
13. 1.0 *M*	$CaCl_2$	
14. 2.0 *M*	$(NH_4)_2HPO_4$	pH = 8.2
15. 3.5 *M*	$(NH_4)_2SO_4$	
16. 1.8 *M*	Li_2SO_4	
17. 2.0 *M*	$MgCl_2$	
18. 2.5 *M*	$MgSO_4$	
19. 2.0 *M*	KH_2PO_4/NaOH	pH = 5.5
20. 2.0 *M*	NaK tartrate	
21. 2.0 *M*	Na_3 citrate (sat)	
22. 5.0 *M*	NaCl	
23. 4.0 *M*	Na formate	
24. 100%	*t*-Butanol	
25. 100%	Ethanol	
26. 100%	Isopropanol	

$$CH_3(CH_2CH_2)_nCH{=}CHCO{-}S{-}CoA + H_2O \leftrightarrow CH_3(CH_2CH_2)_nCHOH{-}CH_2CO{-}S{-}CoA$$

Fig. 2. The reversible stereospecific reactions catalyzed by 2-enoyl-CoA hydratase: 2-*trans*-enoyl-CoA is converted into L-3-hydroxyacyl-CoA; 2-*cis*-enoyl-CoA is converted into D-3-hydroxyacyl-CoA.

Table 3. *Crystallization conditions for 2-enoyl-CoA-hydratase*

Condition	Initial screen		Optimal condition					
1B1	100 m*M*	MES						
	2.5 *M*	Ammonium sulfate						
	10%	Ethanol						
1B4	100 m*M*	MES	100 m*M*	MES	277 K	100 m*M*	MES	277 K
	20%	Ethanol	12%	Ethanol		12%	Ethanol	
	20 m*M*	$CaCl_2$				20 m*M*	$CaCl_2$	
2A3	100 m*M*	Tris	100 m*M*	Tris	293 K			
	2.5%	*t*-Butanol	2.5%	*t*-Butanol				
	30%	MPD	15%	MPD				

= 7.4 and 3 m*M* ethylenediaminetetraacetic acid (EDTA)]. After desalting of the sample, on an Econo-Pac™ 10 DC column (Bio-Rad Laboratories, Richmond, CA) equilibrated with buffer *A*, the 2-enoyl-CoA hydratase was precipitated with 0.14 volumes of ethanol at 266 K. The precipitate was dissolved in buffer *A*, and the purification completed by size-exclusion chromatography on a Superdex 200 HR (10/30) column equilibrated with 200 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH = 7.2, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT) and the runs were carried out with a Smart™ micropurification system (Pharmacia Biotechnology AB, Uppsala, Sweden). The enzyme preparation was stored in 100 m*M* MOPS, 0.5 m*M* EDTA, 0.5 m*M* DTT, 50%(*v*/*v*) glycerol at 253 K.

Results and discussion

2-Enoyl-CoA hydratase was dialyzed overnight against 10 m*M* MOPS, pH = 7.0, 1 m*M* EDTA, 1 m*M* DTT and 1 m*M* sodium azide. The protein was concentrated to 3 mg ml^{-1}, using an Amicon N_2 pressure ultrafiltration unit, with a molecular weight cut-off of 10 kDa. Two sets of crystallization trays were prepared and stored at 277 K and room temperature. Within 2 days small crystals appeared in seven different conditions (Table 1). Different results were obtained at the two different temperatures. Three conditions were chosen for further tests, based on the differences in morphology of the crystals (Table 3). X-ray quality crystals of 2-enoyl CoA-hydratase were grown in the presence of 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) and 12% ethanol or 100 m*M* Tris, 2.5% *tert*-butanol and 15% 2-methyl-2,4-pentanediol (MPD) (Table 3). Both conditions resulted in bipyramidal crystals (longest dimension 0.2 mm, smallest dimension 0.1 mm) with unit-cell dimensions $a = 81$, $b = 81$ and $c = 282$ Å, diffracting to 3 Å. Precession picture data as well as data sets collected with the FAST area detector suggested a primitive, tetragonal space group. A different crystal form was obtained with 20 m*M* calcium chloride, 100 m*M* MES buffer and 12% ethanol (Table 3). The crystals were hexagonal plates with trigonal symmetry, diffracting to 3 Å.

The automated setup of the fast-screening crystallization protocol is now used routinely in our laboratory. Other proteins have been crystallized using this procedure including well diffracting crystals of CSK-SH3 domain and various mutants of trypanosomal triosephosphate isomerase. The flexibility of the system allows a straightforward variation of the composition of the well solutions, for example as suggested by the specific properties of the protein studied or as suggested by the results of the standard initial screening conditions.

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