

Substrate channelling in an engineered bifunctional aldolase/kinase enzyme confers catalytic advantage for C-C bond formation†

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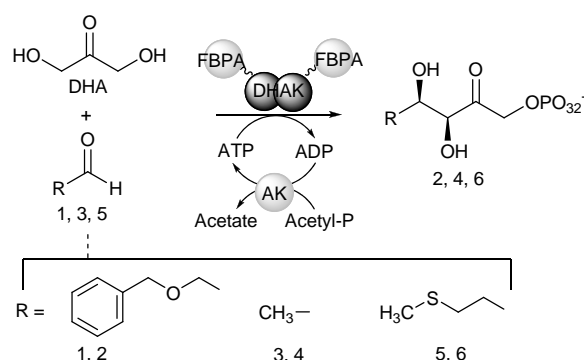
A new bifunctional enzyme that displays both aldolase and kinase activities has been designed and successfully used in the synthesis of aldol adducts, employing DHA as initial donor, with an increase in the reaction rate of 20-fold over the parents enzymes, that can be interpreted in terms of substrate channelling.

The main group of aldolases from the biocatalytic point of view is, arguably, the one that uses dihydroxyacetone phosphate (DHAP) as donor. DHAP-dependent aldolases have been thoroughly used to synthesize carbohydrates, carbohydrate-like structures, or non-carbohydrate compounds.¹ Their main drawback is their strict specificity for the donor substrate. Besides the efforts to overcome the DHAP dependence of aldolases,² an efficient method of DHAP preparation is still essential and several chemical and enzymatic routes of DHAP synthesis have been described in the literature.³

Our research group has developed a straightforward multi-enzyme system for one-pot C-C bond formation.⁴ This route integrates a recombinant ATP-dependent DHA kinase from *Citrobacter freundii* CECT 4626⁵ for *in situ* DHAP formation, fucose-1-phosphate aldolase (F-1PA) for the aldolase catalysed reaction and the regeneration of ATP by acetate kinase (AK). Although this multi-enzyme system is attractive since it is a one-pot/one-step route to the phosphorylated aldol adduct, a considerable number of issues remain. A key point is the number of enzymes that take part in the system because, although the different enzymes can be relatively easily overproduced in recombinant organisms, the purification of proteins is widely recognized to be technically and economically challenging and in general it is the limiting step in bioprocess development.⁶ One way to reduce the number of enzymes to purify is to splice two or more enzymatic activities in only one protein creating a hybrid or fusion enzyme.⁷ The use of fusion proteins in biotransformation may have another, even more interesting effects due to the physical association into covalently linked complexes of enzymes that catalyse sequential reactions. The close proximity of active sites of two enzymes in these multi-enzyme complexes can provide a substantial substrate channelling in addition to the random diffusion path.⁸ Some of the potential catalytic advantages of substrate channelling include the decrease of the transit time required for an intermediate to reach the active site of the next enzyme and the protection of chemically labile intermediates.⁸ In spite of previously exposed, fusion enzymes have been scarcely used in biotransformations in contrast with

their use in other biotechnological fields like protein purification or immobilization of enzymes or receptors for microarrays or biosensors preparation.

Herein, we report the engineering of a new bifunctional enzyme (named DLF) that displays both aldolase and kinase activities in the same polypeptide chain in order to simplify and to improve the catalytic behaviour of the multi-enzyme system described above (Scheme 1).



Scheme 1 Application of the engineered fusion enzyme (DLF) to C-C bond formation using DHA as starting ketone.

A key point at the moment of designing a fusion protein is the possible interactions between subunits that can take place. Since the DHAK from *C. freundii* is a dimeric enzyme,⁹ we choose as aldolase partner the fructose-1,6-bisphosphate (FBP) aldolase from *Staphylococcus carnosus* because of its monomeric structure.¹⁰ In this way, the resulting fusion protein should have a dimeric structure. Both enzymes were fused through a five aminoacid linker. This sequence was designed with the aid of the LINKER program¹¹ to be short enough to keep closer the active sites in order to reinforce the substrate channelling and sufficient flexible to allow the native folding of each protein (see the Supplementary Information).

The fusion enzyme DLF was expressed soluble and retained both activities with a productivity of 135 and 425 U/L of culture broth for the kinase and aldolase activities respectively (ratio kinase:aldolase activities nearly 1:3). Peptide mass fingerprinting verified that purified protein had the DLF expected features and sedimentation equilibrium analysis confirmed that the fusion protein was a homodimer, as the native DHAK (see the Supplementary Information).

Steady-state kinetic analysis of the kinase and aldolase activities in DLF enzyme showed that the fusion of both enzymes did not modified significantly either the K_M or the

turnover number (k_{cat}) of DLF aldolase activity (Table 1). On the other hand, both constants were slightly modified on DLF kinase activity (Table 1).

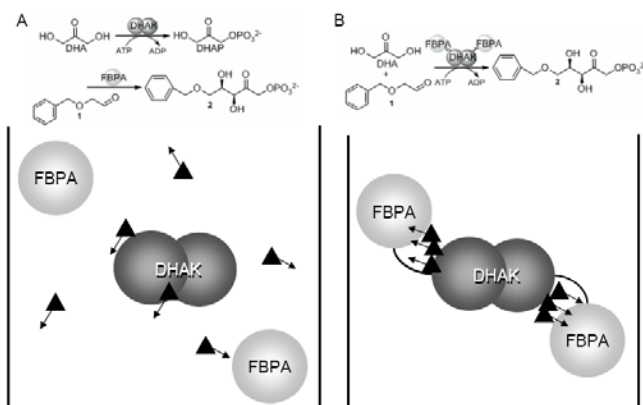
Table 1 Summary of the kinetic constants of the bifunctional DLF and of the native enzymes.

	DHAK ^a	FBPA ^b	DLF	
			kinase	aldolase ^b
K_M (M)	1.22×10^{-6}	1.46×10^{-5}	3.80×10^{-6}	1.88×10^{-5}
k_{cat} (s ⁻¹)	24.13	16.76	8.16	20.30
k_{cat}/K_M (s ⁻¹ /M)	1.98×10^7	1.16×10^4	1.19×10^6	1.08×10^4

^a Constant determined for DHA; data taken from reference 5 ^b Constant determined in retro-aldol reaction with FBP as substrate.

The K_M increase and the k_{cat} decrease of about three times, results in a loss of catalytic efficiency (k_{cat}/K_M) of the kinase activity in the fusion enzyme of about one order of magnitude. This loss in catalytic efficiency could be attributed to the fact that in the fusion enzyme some active centres of the kinase were inaccessible for the substrate. However, despite this decrease, the k_{cat}/K_M value for this activity in the fusion enzyme in the order of 10^6 (s⁻¹/M) is high enough to allow its use in C-C coupling reactions.

To study the proximity or substrate channelling effect, two sets of reactions were run in parallel (Scheme 2). In the first set, the multi-enzyme system formed by a mixture of the native DHAK and FBPA was used to catalyse the condensation between benzyloxyacetaldehyde (**1**) and DHAP. In the second set of reactions the new bifunctional enzyme was employed to catalyse the same reaction. In this study the use of the ATP regeneration system was avoided. Since in the DLF enzyme the kinase:aldolase activities ratio is fixed at 1:3, this same ratio was kept in the assay with the mixture of the native enzymes (see the Supplementary Information).



Scheme 2 Schematic representation of the free diffusion of the intermediate DHAP (▲) in the multi-enzyme system (A) against the possible substrate channelling in the bifunctional fusion enzyme DLF (B).

As it can be observed in Fig. 1, the overall reaction rate was much higher in the reaction catalysed by the fusion enzyme than in the reaction catalysed by the native non-fused enzymes. The overall rate of the coupled reaction catalysed by DLF was $0.41 \mu\text{mol}/\text{min}$ whereas with the multi-enzyme system the overall rate was only of $0.02 \mu\text{mol}/\text{min}$. That is, the physical association of the enzymes produces an increase in the aldol reaction rate of 20-folds.

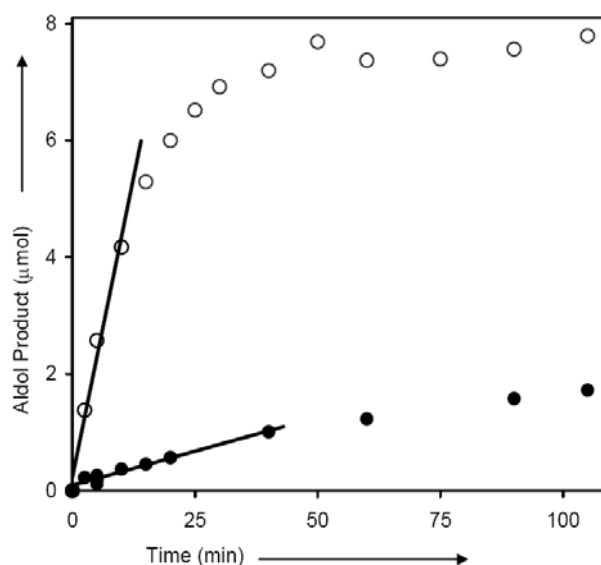


Fig.1 Aldol product formation catalysed by 2.66 U aldolase and 0.91 U kinase using (○) DLF (0.49 mg of protein) or a mixture (●) of DHAK (0.114 mg of protein) and FBPA (0.06 mg of protein). The slope of the straight lines corresponds to the overall rate of aldol product formation.

This observation is consistent with previously reported examples in other fusion enzyme systems,¹² and it cannot be attributed to the different amount of fused enzyme used in terms of mg of protein since this difference was lower than 3 times (see the Supplementary Information). Thus, the results obtained can be interpreted in terms of proximity of the active centres occurred in the fusion enzyme. In an enzymatic reaction sequence the transit time is the time required for a metabolite to reach the next enzyme in the pathway. Transit time must be related with the time of diffusion from one active site to the other and is expected to be in the order of r_E^2/D_s , where r_E is the average separation distance of enzyme molecules and D_s is the diffusion coefficient of the substrate.¹³ Thus, DHAP produced by the kinase should have a lower transit time to the FBPA active sites in the fusion proteins compared with that of the non-fused enzyme system since r_E has been significantly reduced. Another possible explanation for the obtained results can be deduced from an increased local concentration of the intermediate DHAP. Nevertheless, in both cases free diffusion of DHAP to the bulk solution is limited because the active centres proximity in the fused enzyme.

A preliminary study of the synthetic applicability of this new bifunctional enzyme in the stereoselective C-C bond formation was conducted in small scale reactions using DHA as starting ketone and benzyloxyacetaldehyde (**1**), acetaldehyde (**3**) and 3-(methylthio)propionaldehyde (**5**) as acceptors (Scheme 1). The percentage of aldol adducts obtained after 20 h. of reaction with the different aldehydes (Table 2) was of 58.2 for benzyloxyacetaldehyde (**1**), 82.6 with acetaldehyde (**3**) and 67.3 with (methylthio)propionaldehyde (**5**). Formation of aldol products was established by NMR analysis (see the Supplementary Information). In a first approximation to determine if the stereoselectivity of the aldol reaction had been modified by

the fusion process, we analysed the stereoisomers formed in the DLF catalysed reactions applying the enzymatic assay described by Sheldon and co-workers based on the reversibility of the aldol reaction.¹⁴ In general, the results summarized in Table 2 are in agreement with those reported by Sheldon and co-workers: the aldol reaction catalysed by DLF is highly, but far from absolutely, stereospecific for products with D-threo configuration and it is strongly influenced by the acceptor. In particular, the data regarding acetaldehyde (**3**) are not dissimilar to those reported for the same aldehyde by Sheldon and co-workers.¹⁴ Therefore, we can conclude that the fusion process has not influence the stereoselectivity of the aldol reaction.

Table 2 Conversion and ratio of stereoisomeric products formed in DLF catalysed reactions.^a

Acceptor	Conversion (%)	Stereoisomer (%) ^b		
		D-threo	L-threo	D-erythro
1	58.2	79.4	2.1	18.5
3	82.6	71.8	2.0	26.2
5	67.3	96.0	0	4.0

^a Only stereoisomers with D-threo, L-threo and D-erythro configuration could be detected. ^b Percentage was calculated with respect to the total aldol adduct formed.

In conclusion, we have developed a fusion enzyme which consists of monomeric fructose-1,6-bisphosphate aldolase from *S. carnosus* and the homodimeric dihydroxyacetone kinase from *C. freundii* CECT 4626 with an intervening five aminoacids linker. The fusion protein was expressed soluble and retained both kinase and aldolase activity with a so good catalytic efficiency. The proximity of the active centres in the fusion enzyme promotes a kinetic advantage as indicate the 20-fold increment in the overall rate of the aldol reaction. Finally, it seems that the fusion has not modified the steric course of the aldol reaction catalysed by the bifunctional DLF enzyme.

Notes and references

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[†] Electronic Supplementary Information (ESI) available: Materials, detailed experimental procedures and NMR characterization of compounds **2**, **4** and **6**. See DOI: 10.1039/b000000x/

[‡] We adopt here the operational definition of substrate channelling given by Ovádi, according to which the term “substrate channeling” designates the coupling of two or more enzymatic reactions in which the reaction product of one enzyme is transferred to the next enzyme without escaping into the bulk phase. For reviews on substrate channelling and its metabolic significance see reference 8.

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Substrate Channelling in an Engineered Bifunctional Aldolase/Kinase Enzyme Confers Catalytic Advantage for C-C Bond Formation.

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Materials and General Procedures

Staphylococcus carnosus CECT 4491 was provided from the Spanish Type Culture Collection (CECT). *E. coli* BL21(DE3) competent cells were purchased from Stratagene Co. (San Diego, CA). Restriction enzymes, *Taq* polymerase and T4-DNA ligase were purchased from MBI Fermentas AB (Lithuania). Triosephosphate isomerase (TIM), α -glycerophosphate dehydrogenase (α GDH), lysostaphin and acetate kinase (AK), were purchased from Sigma-Aldrich (St. Louis, MO). PCR primers were purchased from Isogen Life Science (Spain) and the pET-28b(+) expression vector was purchased from Novagen. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Applichem GmbH (Germany). Plasmids and PCR purification kits were from Promega (Madison, WI) and DNA purification kit from agarose gels was from Eppendorf (Hamburg, Germany). SDS-PAGE was performed using 10% and 5% acrylamide in the resolving and stacking gels, respectively. Gels were stained with Coomassie brilliant blue R-250 (Applichem GmbH, Germany). Electrophoresis was always run under reducing conditions, in the presence of 5% β -mercaptoethanol. Protein and DNA gels were quantified by densitometry using GeneGenius Gel Documentation and Analysis System (Syngene, U.K.). Nickel-iminodiacetic acid (Ni^{2+} -IDA) agarose was supplied by Agarose Bead Technologies (Spain). Size-exclusion chromatography was carried out on a HiLoad 26/60 Superdex 75 PG column controlled using the AKTA-FPLC system (GE Healthcare Life Science). All other chemicals were purchased from commercial sources as reagent grade.

Cloning, overexpression and purification of FBPA from *S. carnosus*

DNA manipulation was according to standard procedures.¹ DNA template for amplification of the *fda* gene was obtained from the *S. carnosus* strain CECT 4491. The oligonucleotides 5'-ATATTCATATGAACCAAGAACAACAATTTGAC-3' and 5'-TATTACTCGAGTTAAGCTTTGTTTACTGA-3' were used as leftward and rightward primers respectively (the recognition sequence for *Nde*I and *Xho*I are underlined). To extract the DNA, *S. carnosus* cells was suspended in lysis buffer containing Tris 10 mM, EDTA 10 mM, lysozyme 200 U/mL and lysostaphin 25 U/mL.² PCR amplification was performed in a 10 μ l reaction mixture and subjected to 25 cycles of amplification. The cycle conditions were set as follows: denaturation at 94°C for 1 min, annealing at 55 °C for 2 min and elongation at 72 °C for 1 min. The purified PCR product was digested with *Nde*I and *Xho*I and ligated into the doubled digested vector pET-28b(+) to yield the plasmid pET-*fda*. This plasmid was transformed into *E. coli* BL21(DE3) competent cells.

A colony containing the plasmid pET-*fda* was cultured in Luria-Bertani (LB) broth containing kanamycin (26 μ g/mL) at 37 °C with shaking. When the culture reached an O.D_{600nm} of 0.5-0.6, FBPA expression was induced with IPTG (0.4 mM) and the temperature was dropped to 30 °C. The culture was maintained O/N. After that, the culture was centrifuged at 3,000 x g during 30 min at 4 °C and the resulting pellet was treated with lysozyme and DNase for protein extraction.³

The recombinant protein containing an N-terminal 6xHis tag was purified in a Ni^{2+} -IDA-agarose column pre-equilibrated with sodium phosphate buffer (20 mM, pH 7.5). FBPA was eluted with the same buffer containing imidazole 1 M. All the fractions containing protein were pooled together and further purified by size-exclusion chromatography on a HiLoad 26/60 Superdex 75 PG column controlled using the AKTA-FPLC system (GE Healthcare Life Science). The column was

¹ J. Sambrook, E. F. Fritsch, T. Maniatis *Molecular cloning. A laboratory manual*. 1989. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y

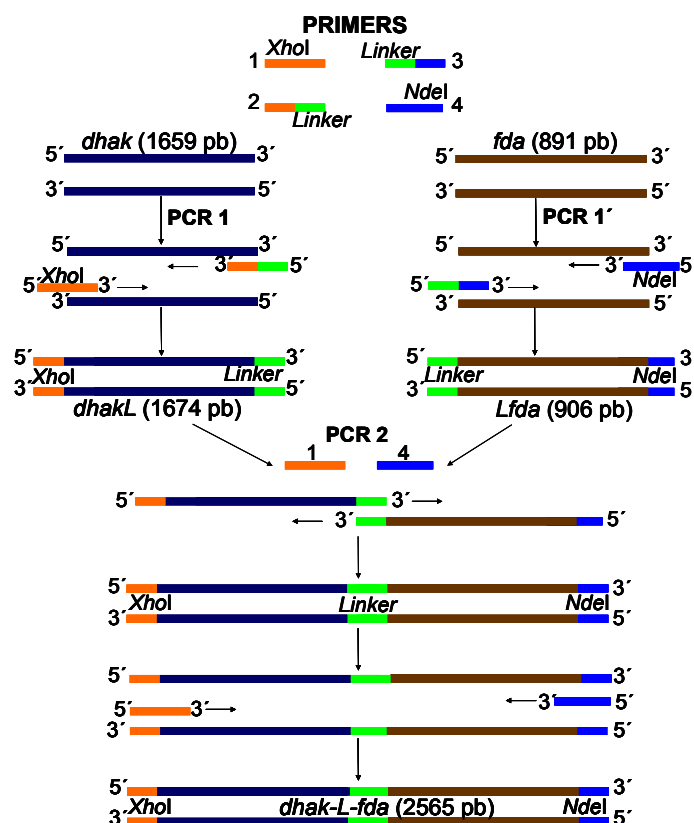
² M. Ligozzi, R. Fontana, *Afr J Biotechnol* 2003, 2, 251-253.

³ A. Bastida, A. Fernández-Mayoralas, R. Gómez, F. Iradier, J. C. Carretero, E. García-Junceda, *Chem. Eur. J.* 2001, 7, 2390-2397

developed in 50 mM phosphate buffer pH 7.2 containing NaCl (0.15 M) at a constant flow rate of 1.0 mL/min

Construction, expression and purification of the bifunctional DLF enzyme

The *dhak-l-fda* fusion gene was constructed by gene splicing by overlap extension (SFigure 1).⁴ This method comprises two PCR steps. In the first PCR step, the *dhak* gene from *C. freundii* and the *fda* gene from *S. carnosus* were amplified separately including sites for the restriction enzymes *Nde*I (5'-*dhak*) and *Xho*I (3'-*fda*). Extremes 3'-*dhak* and 5'-*fda* included the 15 nucleotides linker sequence (*l*). These amplified genes were used as templates for a second PCR step.



SFigure 1. Schematic representation of DHAK and FBPA genes splicing by overlap extension.

The sequences of the primers used are shown in the STable 1. The first two PCRs amplification were performed in 10 µl reaction mixture and subjected to 25 cycles of amplification. The cycle conditions were set as follows: denaturation at 94°C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 2 min. A 1:1 mixture of the purified PCR products was used as template in the second PCR step. The reaction conditions were identical to the previously described, except for the elongation time that was 3 min. After purification, the *dhak-l-fda* fragment was digested with *Nde*I and *Xho*I and ligated into the doubled digested vector pET-28b(+) to yield the plasmid pET-*dhak-l-fda*. This plasmid was transformed into *E. coli* BL21(DE3) competent cells.

Procedure for expression and purification of the bifunctional enzyme were identical to those described for the FBPA enzyme except in the IMAC purification step. In this case, previously to elute the enzyme with imidazole 1 M, the column was washed with 10 volumes of sodium phosphate buffer (20 mM, pH 7.5) containing imidazole 50 mM.

⁴ R. M.Horton, H. D. Hunt, S. N. Ho, J. K. Pullen, L. R. Pease, *Gene*, **1989**, 77, 61-68.

STable 1. Sequences of the primers used to splice the *dhak* and *fda* genes. The endonuclease recognition sequences are in bold and the linker sequence underlined

Fragment	Primer	Sequence	Endonuclease
<i>dhak-l</i>	NtNdhak	5'-ATATT CATATG TCTCAATTCTTTTTT-3'	<i>NdeI</i>
	CtFdhak	5'- <u>CTGGCCCTGGCCCTGGCC</u> CAGCTCACTCTC-3'	-
<i>l-fda</i>	CtScXfda	5'-TATTACT CGAG TTAAGCTTTGTTTACTGA-3'	<i>XhoI</i>
	NtFScfda	5'- <u>CAGGGCCAGGGCCAGA</u> ACCAAGAACAATTTGACAAA-3'	-
<i>dhak-l-fda</i>	NtNdhak	5'-ATATT CATATG TCTCAATTCTTTTTT-3'	<i>NdeI</i>
	CtScXfda	5'-TATTACT CGAG TTAAGCTTTGTTTACTGA-3'	<i>XhoI</i>

Protein analysis

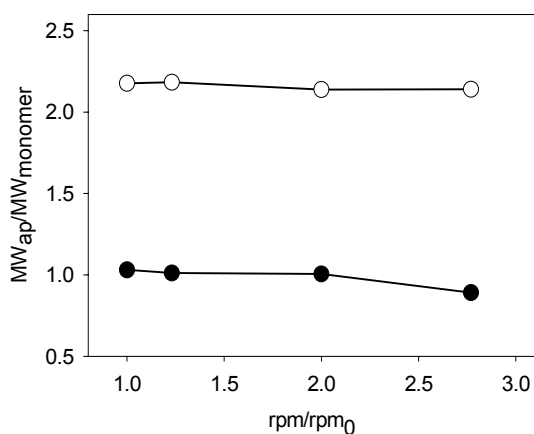
Amino acid analyses of purified recombinant proteins were performed in the Protein Chemistry Service of the Centre of Biological Research (CIB-CSIC) to determine the protein concentration. The absorption spectrum of different quantified samples allowed determination of the molar extinction coefficient at 280 nm for recombinant FBPA ($\epsilon^{280} = 46292 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and for DLF ($\epsilon^{280} = 77928 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Peptide mass fingerprint analyses (SFigure 2) from the SDS-PAGE band corresponding to the putative FBPA and DLF were performed at the Proteomic Unit of the Spanish National Center of Biotechnology (CNB-CSIC). Samples were digested with sequencing grade trypsin overnight at 37°C. The analysis by MALDI-TOF mass spectrometry produces peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. Data were collected in the m/z range of 800-3600. 20 peptides covering the major part of the amino acid sequence of FBPA were identified (SFigure 2A). Almost all the predicted tryptic peptides with molecular masses falling in the analyzed m/z range were found in the peptide mass fingerprint of the recombinant FBPA.

**SFigure 2.** Peptide mass fingerprint of FBPA from *S. carnosus* (A) and bifunctional DLF (B). The sequence of the identified peptides are shaded and underlined. Molecular mass of each peptide is indicated in Da. Linker sequence in the DLF enzyme is marked in yellow.

In the case of the DLF peptide mass fingerprinting, 20 peptides covering the DHAK sequence were identified and 13 peptides covering the FBPA sequence were also identified (SFigure 2B). Peptide mass fingerprinting verified that purified proteins had the expected features of FBPA and DLF respectively.

Sedimentation equilibrium experiments were performed at the Department of Chemical Physics of Biological Macromolecules (Institute of Chemical Physics "Rocasolano"; CSIC). The initial concentration of the protein used in these experiments was 0.96 mg/mL. These experiments confirmed the expected molecular weight and showed that the fusion protein was a homodimer, as the native DHAK (SFigure 3).



SFigure 3. Sedimentation equilibrium analysis of FBPA (●) and DLF (○).

Enzyme activity assays

Phosphorylation of DHA was measured spectrophotometrically in a coupled enzymatic assay as previously described.⁵ Aldolase activity was spectrophotometrically measured by the retro-aldol reaction using fructose-1,6-bisphosphate (FBP) as substrate.⁶ The aldolase activity assays were run at room temperature following the decrease of absorbance at 340 nm ($\epsilon_{NADH}^{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$) for 5 minutes in 1 mL reaction mixture containing Tris-HCl buffer (40 mM, pH 8.0), NADH (0.2 μmol), α -GDH/TIM (2 U), 1.0 μmol of FBP and the bifunctional DLF or FBPA.

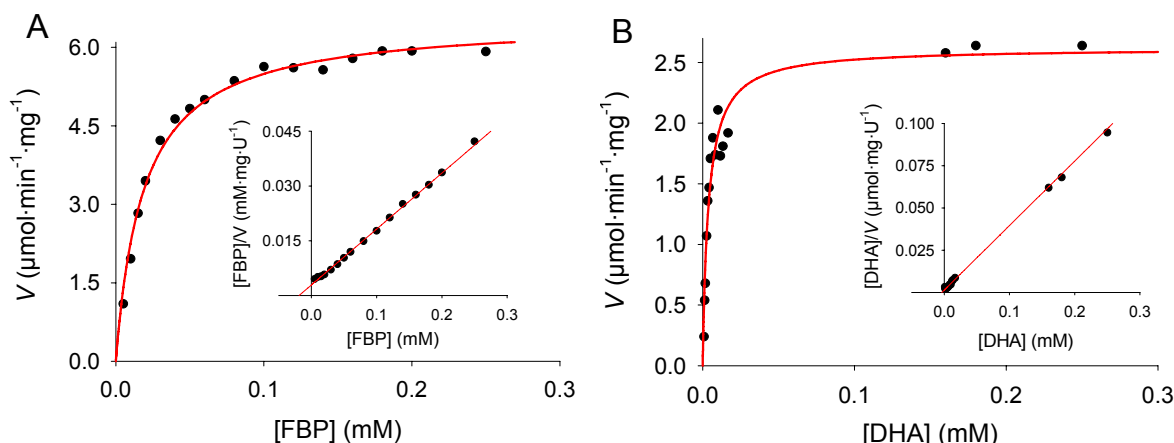
Steady-state kinetic assays

Steady-state kinetic assays with DLF were measured at 25 °C in 96-well plates in a total volume of 0.3 mL. Measurements of kinetic parameters for FBP (SFigure 4A) were performed with 5.3 $\mu\text{g/mL}$ of purified protein at sixteen different FBP concentrations in the range 0.005-0.25 mM. Assays to determine the kinetic parameters for DHA (SFigure 4B) were performed with 24 $\mu\text{g/mL}$ of purified DLF at twelve concentrations of substrate under saturating concentrations of $[\text{MgATP}]^{2-}$ complex (3.75 mM).

The catalytic constant (k_{cat}) is the result of dividing the V_{max} (in units of $\text{M} \times \text{time}^{-1}$) by the concentration (Molar) of enzyme.

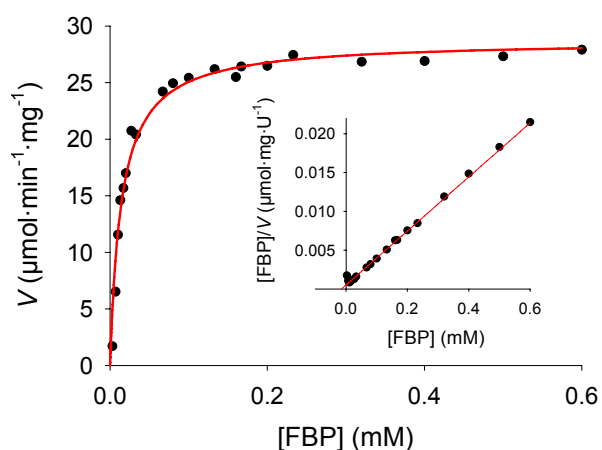
⁵ I. Sánchez-Moreno, L. Iturrate, R. Martín-Hoyos, M. L. Jimeno, M. Mena, A. Bastida, E. García-Junceda, *ChemBioChem*, DOI:10.1002/cbic.200800573.

⁶ H. U. Bergmeyer *Methods of Enzymatic Analysis* vol. 2, 3rd ed.; (1984) Verlag Chemie: Deerfield, FL.



SFigure 4. Substrate kinetics of aldolase (A) and kinase (B) activities for fusion enzyme DLF. The inserts show Hanes-Woolf plots used for kinetic constants determination.

Steady-state kinetic assays with FBPA were measured as well at 25 °C in 96-well plates in a total volume of 0.3 mL. Measurements of kinetic parameters for FBP (SFigure 5) were performed with 0.83 μg/mL of purified protein at twenty different FBP concentrations in the range 0.005-0.6 mM. Kinetic constants were obtained using the built-in nonlinear regression tools in SigmaPlot 8.0. For the determination of apparent kinetic constants (variation of only one substrate), initial velocities (V_i) were fitted to the Michaelis-Menten equation



SFigure 5. Substrate kinetic for FBPA from *S. carnosus*. The insert shows Hanes-Woolf plot used for kinetic constants determination.

Proximity Effect study

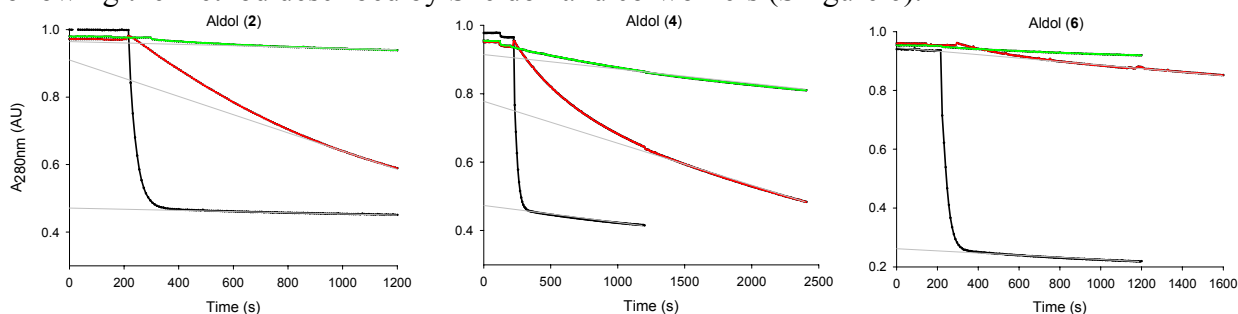
To study the proximity effect, the rates of the coupled reaction catalysed by DLF or a combination of the two native enzymes were measured and compared under same conditions. In both cases, 0.91 U of kinase activity and 2.66 U of aldolase activity were used. To fit the Units of activity was necessary to use a slightly higher amount of DLF in terms of mg of protein. Thus, 0.49 mg of DLF was used by a total of 0.175 mg for the sum of DHAK (0.114 mg) and FBPA (0.061). The reactions were carried out at room temperature in 1.5 mL of phosphate buffer (20 mM, pH 7.5) containing 0.05 mmol of DHA, 0.15 mmol of benzyloxyacetaldehyde (**1**), 12.5 μmol of MgSO₄ and 12.5 μmol of ATP. At different time, 50 μL aliquots were taken. The reaction was stopped with HClO₄ (7%) and the aldol product formed quantified by the retro-aldol assay.

Synthetic application of the bifunctional DLF enzyme

C-C bond formation reactions catalysed by the fusion enzyme DLF, were carried out at room temperature in 1.5 mL of phosphate buffer (20 mM, pH 7.5) containing 0.05 mmol of DHA, 0.15 mmol of aldehyde acceptor (benzyloxyacetaldehyde (**1**), acetaldehyde (**3**) and 3-(methylthio)propionaldehyde (**5**) respectively), 0.1 mmol of acetyl phosphate, 12.5 μ mol of MgSO_4 , 3 U of AK and DLF (1.5-2 U and 3-6 U of kinase and aldolase activities respectively). The reactions begin with the addition of 3.4 μ mol of ATP. When the consumption of DHA was higher than 90 % (~20 h), the reactions were stopped and passed through activated carbon. The eluted was freeze-dried for NMR characterization.

Stereochemistry study of aldol reactions catalyzed by DLF enzyme

The determination of the stereoisomeric products formed by bifunctional DLF was carried out following the method described by Sheldon and co-workers (SFigure 6).⁷

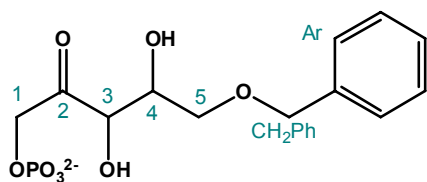


SFigure 6. Enzymatic determination of stereoisomers of aldol products (**2**), (**4**) and (**6**). Plots show retro-aldol reactions catalysed by RAMA (black line), Fuc-IPA (red line) and Rham-IPA (green line).

NMR procedures.

^1H and ^{13}C NMR spectra, using D_2O as solvent, were recorded on a Varian SYSTEM 500 spectrometer equipped with a 5 mm HCN cold probe with field z-gradient, operating at 500.13 and 125.76 MHz for ^1H and ^{13}C , respectively. The sample temperature was maintained constant at 298 K. One-dimensional NMR experiments were performed using standard Varian pulse sequences. Two-dimensional [^1H , ^1H] NMR experiments (gCOSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 3000 Hz in both dimensions, 4096 complex points in t_2 and 4 transients for each of 256 time increments, and linear prediction to 512. The data were zero-filled to 4096×4096 real points. Two-dimensional [^1H - ^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^1H spectral window, a ^{13}C spectral windows of 15 000 Hz, 1 s of relaxation delay, 1024 data points, and 256 time increments, with a linear prediction to 512. The data were zero-filled to 4096×4096 real points. Typical numbers of transients per increment were 4 and 16, respectively.

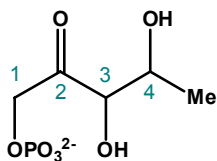
5-(benzyloxy)-3,4-dihydroxy-2-oxopentyl phosphate (2)



⁷ R. Schoevaart, F. v. Rantwijk, R. A. Sheldon, *Biotechnol. Bioeng.* **2000**, 70, 349-352

$^1\text{H NMR}$ (500 MHz, D_2O , 298 K): δ 7.2-7.1 (m, 5H, Ar), 4.52 (dd, 1H, $J=18.5, 5.8$ Hz, H-1_A), 4.40 (dd, 1H, $J=18.5, 5.8$ Hz, H-1_B); 4.4-4.3 (m, 2H, CH_2Ph), 4.29 (s, 1H, H-3), 4.15-4.05 (m, 1H, H-4), 3.50-3.45 (m, 1H, H-5_A), 3.45-3.40 (m, 1H, H-5_B).

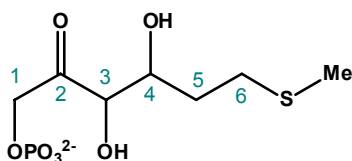
3,4-dihydroxy-2-oxopentyl phosphate (**4**)



$^1\text{H NMR}$ (500 MHz, D_2O , 298 K): δ 4.55 (dd, 1H, $J=18.8, 6.1$ Hz, H-1_A), 4.45 (dd, 1H, $J=18.8, 6.1$ Hz, H-1_B); 4.20 (d, 1H, $J=2.6$ Hz, H-3); 4.14 (dq, 1H, $J=6.4, 2.4$ Hz, H-4), 1.10 (d, 3H, $J=6.3$ Hz, Me).

$^{13}\text{C NMR}$ (125 MHz, D_2O , 298 K): δ 211.6 (C-2), 78.5 (C-3), 67.9 (C-4), 67.7 (C-1), 18.4 (Me).

3,4-dihydroxy-6-(methylthio)-2-oxohexyl phosphate (**6**)



$^1\text{H NMR}$ (500 MHz, D_2O , 298 K): δ 4.55 (dd, 1H, $J=18.8, 6.6$ Hz, H-1_A), 4.45 (dd, 1H, $J=18.8, 6.6$ Hz, H-1_B); 4.28 (d, 1H, $J=2.2$ Hz, H-3); 4.05 (ddd, 1H, $J=14.4, 11.5, 9.2$ Hz, H-4), 2.5-2.4 (m, 2H, H-6), 1.95 (s, 3H, Me), 1.8-1.7 (m, 2H, H-5).

$^{13}\text{C NMR}$ (125 MHz, D_2O , 298 K): δ 211.5 (C-2), 77.7 (C-3), 70.4 (C-4), 68.1 (C-1), 29.6 (C-6), 23.5 (C-5); 14.3 (Me).