

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Doctor

José Luis Salazar

Director de Nuevas Creaciones (E)

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. _____ S. _____ D. _____

Referencia: Expediente No. 06.009.419

Solicitud de Patente de Invención titulada: "Polipéptidos cristalinos del receptor 2 del factor de necrosis de tumor"

Solicitante: Amgen Inc.

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 11**DEL 14 DE ENERO DE 2011, OFICIO NO. 0627 (EXAMEN DE FONDO)**

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término de prórroga solicitado en mi memorial 06-009419-00011-0000 del 8 de Abril de 2011:

1. Objeciones del Examinador**1.1 Claridad de la solicitud**

Su Despacho enuncia en forma resumida *"que no se describe las características técnicas del cristal. Se establece también que en la descripción únicamente se dan formas de realización de los cristales, pero no se evidencian las ventajas comparativas, ni el efecto técnico que resuelve el problema de estabilidad de los compuestos reivindicados, y que el capítulo reivindicatorio presenta falta de suficiencia en la descripción, por cuanto no existe información para que un técnico versado en la materia pueda llevar a cabo la invención.*

Se menciona además, *“que las reivindicaciones de proceso tampoco describen las características técnicas del mismo....Tampoco existen datos comparativos (tales como DSC) que permitan determinar las propiedades de los compuestos con relación a otros en forma nativa.*

Con relación a las reivindicaciones 21 y 23 se establece que no está correctamente definidas dado que caracterizan el cristal y la composición por su proceso y no por las características técnicas.

En referencia a la reivindicación 22 se menciona que no define la composición conforme a sus características técnicas, es necesario tener en cuenta que una composición presenta más de un compuesto.

Por último, su Despacho objeta las reivindicaciones 24 a 27 relacionada con el uso de los compuestos reivindicados para la preparación del medicamento.

1.2 Novedad

El Examinador plantea que, después de realizada la búsqueda de anterioridades, encontró documentos que afectan la novedad de las reivindicaciones 1 a 23 de la presente solicitud.

Menciona los documentos **D1** (WO 02/072636), **D2** (Jen y Merkle) y **D3** (Yang et al,) como documentos relacionados con la solicitud.

Sin embargo, el Examinador considera que solo el documento **D1** (WO 02/072636) afecta la novedad de la presente solicitud por cuanto:

“D1 enseña una forma de realizar cristales de anticuerpos y fragmentos de los mismos. Dicha metodología, incluye cristales de etanercept sus formas de realización que incluyen las sales presentes en la solicitud y agentes buffer.

Los elementos característicos de las composiciones reivindicadas, ya eran conocidos como se observa en D1, la forma de realizar los cristales y las posibles sales para su preparación así como también los resultados esperados. Por tal razón los compuestos reivindicados no pueden ser considerados novedosos”.

2. Respuesta a las observaciones del examinador.

2.1 Modificación

Con el fin de atender a las observaciones del examinador y de conformidad con el artículo 34 de la Decisión 486, que permite al solicitante modificar la solicitud en cualquier momento del trámite sin que se amplíe el alcance de la invención inicialmente presentada, me permito presentar un nuevo pliego reivindicatorio conformado por 19 reivindicaciones.

En particular, se modificó las reivindicaciones independientes 1 y 6 al incluirse que el etanercept está en la forma de una barra o de una aguja.

Por otro lado, se cancelaron las reivindicaciones 2, y 21 a 27.

2.2 Claridad de la solicitud

Como se mencionó anteriormente, con el fin de atender a las observaciones de su Despacho se presenta un pliego reivindicatorio modificado.

Por otro lado, mi representada argumenta que una persona medianamente versada en la materia sería capaz de llevar a cabo la invención tal como se reivindica. Por favor, nótese que las reivindicaciones modificadas se relacionan con cristales de etanercept en la forma de barra o agujas (Por favor, ejemplos 1 y 2 de la descripción) y métodos para fabricarlos.

Aquellos medianamente versados en el campo de cristalización y basados en la descripción de la solicitud, al menos en la página 16, línea 29- página 17, línea 17 del texto en inglés y en las páginas 43 y 44 y ejemplos 1 y 2 del texto en español serían capaces sin ninguna ambigüedad de entender la característica de una forma de cristal de etanercept (en forma cristalina) e identificarla utilizando tecnología comúnmente disponible.

2.2 Novedad:

La novedad de una solicitud está legalmente definida en el Artículo 16 de la Decisión 486

así: “una invención se considerará nueva, cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso de la prioridad reconocida...”

En virtud de la anterior definición, consideramos que el documento **D1** no constituye documento del arte previo que afecte la novedad de la presente solicitud por las siguientes razones:

En primer lugar, las reivindicaciones modificadas que se presentan junto con este escrito son novedosas con respecto a **D1** ya que **D1** no revela cristales de Etanercept. El voluminoso documento **D1** (que trata la cristalización, y la estabilización del cristal de rituximab y trastuzumab) menciona etanercept solamente en tres oportunidades:

En la Página 19 (líneas 3-7): “Antibodies which may be crystallized according to the present invention incluye, for example, Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept and Ibritumomab tiuxetan”. El subrayado fuera de texto.

En español: “Anticuerpos que pueden cristalizarse de acuerdo a la presente invención, incluyen, (por ejemplo, Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept y Ibritumomab tiuxetan). Subrayado fuera de texto.

En la página 99 (líneas 2-27): “Such antibodies include, but are not limited to, commercially available antibodies, including, but not limited to: (1) Abciximab (ReoPro) (antiGPI/IIb/IIIa receptor; for the treatment of cardiovascular disease) (Centocor, Leiden, The Netherlands); [. . .] (8) Etanercept (ENBREL™) (inflammatory disease) (Immunex, Seattle, WA; (9) Ibritumomab tiuxetan (Zevalin™) (radioimmunotherapy for cancer) (IDEC Pharmaceuticals, San Diego, CA).” El subrayado fuera de texto.

En español:

“Tales anticuerpos incluyen pero no están limitados a, anticuerpos disponibles

comercialmente, incluyendo, pero no limitado a: (I) Abciximab (ReoPro) (antiGPI/IIb/IIIa receptor; para el tratamiento de enfermedad cardiovascular) (Centocor, Leiden, The Netherlands); [. ..] (8) Etanercept (ENBREL™) (enfermedad inflamatoria) (ImmuneX, Seattle, WA); (9) Ibritumomab tiuxetan (Zevalin™) (radioinmunoterapia para el cáncer) (IDEC Pharmaceuticals, San Diego, CA)". Subrayado fuera de texto.

En la reivindicación 14 : "The crystal according to anyone of claims 1, 2 or 3, wherein the antibody is selected from the group consisting of: Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept, and Ibritumomab tiuxetan. ". Subrayado fuera de texto.

En español.

Reivindicación 14: "El cristal de acuerdo a cualquiera de las reivindicaciones 1, 2 o 3 en donde el anticuerpo se selecciona del grupo que consiste de: Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept, and Ibritumomab tiuxetan. " Subrayado fuera de texto.

Sin embargo, debe notarse que el Etanercept no es un anticuerpo. La molécula es una proteína de fusión que comprende el receptor TNF unida al fragmento Fc de IgG1. Por lo tanto, la revelación de **D1** con respecto al etanercept y a sus cristales es claramente erróneo.

Al parecer, el etanercept fue incluido erróneamente en la lista de anticuerpos arriba citada debido de alguna forma a sus usos terapéuticos similares, la persona medianamente versada en la materia puede darse cuenta de esto.

Por favor, nótese además que estructuralmente, la proteína de fusión de Etanercept es esencialmente diferente a las estructuras de cualquiera de los anteriores anticuerpos, que, excepto por una parte del Fc potencialmente similar, no tiene nada en común con el Etanercept.

Por lo tanto, **D1** debe descartarse como anterioridad para evaluar la novedad de la materia objeto de las reivindicaciones modificadas de la solicitud en estudio.

2.3 Nivel inventivo

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *“si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”*.

Aunque su Despacho no efectuó observaciones con respecto al requisito de nivel inventivo, mi representada presenta los siguientes argumentos con el fin de agilizar el trámite de la solicitud en estudio.

Las presentes reivindicaciones son inventivas con relación a los documentos **D1**, **D2** y/o **D3**. **D2** y/o **D3** no remedian las deficiencias de **D1**.

D2 es una publicación científica, en la cual se discuten las ventajas de cristales de proteína y se revela la posible utilidad de la cristalización en la formulación de proteínas terapéuticas.

Específicamente, como se indicó en la parte de la introducción de la presente solicitud, se afirma, que cristales de proteína han mejorado el manejo de la estabilidad física y las propiedades de liberación sostenidas liberadas. Sin embargo, no se hace ninguna mención de etanercept en **D2**.

Además, se menciona específicamente que:

“good crystal drug candidates should have sufficient physical robustness that allows handling as a solid dosage form”.

En español: *“buenos candidatos de cristales de fármacos deberían tener suficiente robustez física que permita su manipulación a una dosificación en forma sólida”*

Y que esto generalmente no es el caso con cristales de proteína frágiles (por favor, ver página 1486, columna derecha, al comienzo del segundo párrafo).

Además, en el resumen concluyente de **D2** (página 1487), se establece específicamente que del planteamiento revelado en **D2** no puede ser posible para todos los candidatos de proteína porque los cristales robustos y estables físicamente puede ser evasivos. Por lo tanto, cada proteína individual debe ser examinada separadamente. Aunque la persona medianamente versada en la materia pueda haber entendido que la cristalización puede ser posible para

algunas proteínas, no habría ninguna indicación que fuera posible para Etanercept.

Lo anteriormente expuesto aplica para **D3**. Dicho documento describe la cristalización de tres anticuerpos monoclonales, rituximab, trastuzumab y infliximab. En dicho documento se desarrolló exitosamente un nuevo proceso para la producción de lotes grandes de anticuerpos monoclonales de anticuerpos, y uno de los anticuerpos cristalizados producidos, infliximab, se conoce que es un inhibidor TNF. Sin embargo, el hecho de que tanto el etanercept y el infliximab son inhibidores TNF no habrían sido considerados relevantes para una persona medianamente versada en la materia en el contexto de cristalización.

La persona medianamente versada en la materia al leer **D3** en el contexto de los otros documentos arriba discutidos no habría tenido ninguna indicación de hasta donde el proceso del documento **D3** podría aplicarse exitosamente al etanercept. La única similitud estructural entre el infliximab y el etanercept son que ambas proteínas tienen de alguna manera un tamaño similar y comprenden una región Fc.

Sin embargo, las secuencias de los aminoácidos son considerablemente diferentes. Tal como se evidencia en la publicación de Longenecker¹ (ver copia adjunta), aún cambios en pequeños aminoácidos pueden ser decisivos con respecto a la posibilidad para formar cristales.

Al considerar las principales diferencias estructurales entre el etanercept y el infliximab, es evidente que el éxito de la cristalización del infliximab no habría sido un valor previsible para la pregunta si la cristalización sería posible para etanercept.

Además, el patrón de la glicosilación en el etanercept es bastante diferente del encontrado en un anticuerpo por lo que es altamente incierto si una proteína tal como el etanercept podría del todo cristalizarse. Se evidencia el hecho de que las diferencias en el patrón de glicosilación de las proteínas pueda dramáticamente afectar la posibilidad para obtener cristales de proteína. Por favor, ver el documento de White² et al que se anexa junto con este

¹ Protein crystallization by rational mutagenesis of surface residues: Lys to Ala mutations promote crystallization of RhoGDI, Longenecker et al.

² Biological activity, membrana-targeting modification, and crystallization of soluble human decay accelerating factor expressed in E. coli, Jennifer White et al, 2004.

escrito. (ver lo que se establece en la página 2407, columna derecha, segundo párrafo).

Por último, no hay ninguna sugerencia en **D3** para aplicar el proceso revelado allí a otros tipos de polipéptidos. Por lo tanto, **D3** no proporcionaría ninguna motivación a la persona medianamente versada en la técnica ni mucho menos de una expectativa razonable del éxito con relación a la cristalización de etanercept.

Por lo anteriormente expuesto, no habría sido obvio para una persona medianamente versada en la materia intentar cristalizar el etanercept. Además, la persona versada no habría tenido una expectativa razonable de lograr obtener una composición de etanercept mejorado por cristalización.

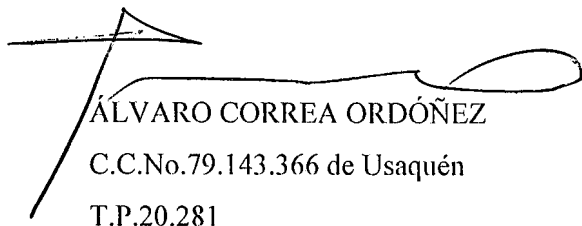
En este orden de ideas, tenemos que la invención propuesta por el solicitante cumple con los requisitos de patentabilidad y de esta manera es susceptible de ostentar el título de patente de invención.

3. Solicitudes concedidas en otros países para esta misma invención.

A manera informativa, pongo en conocimiento del Examinador que la patente US 7,276,477 equivalente a la presente solicitud colombiana ha sido otorgada. Aún cuando entiendo que esta decisión de ninguna manera obligan al Despacho para decidir de igual forma, considero que por tratarse de una pregunta fáctica la determinación de la existencia de novedad, nivel inventivo y aplicación industrial, las decisiones de otras oficinas de patentes constituyen un antecedente persuasivo en la materia.

En virtud de lo anterior, solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,



ÁLVARO CORREA ORDÓÑEZ
C.C.No.79.143.366 de Usaquén
T.P.20.281

Anexos:

- Capítulo reivindicatorio modificado conformado por 19 reivindicaciones.
- Copia de la carátula y reivindicaciones de la Patente US 7,276,477.
- Artículo: "Protein crystallization by racional mutagenesis of surface residues: Lys to Ala mutations promote crystallization of RhoGDI"
- Artículo: "Biological activity, membrane-targeting modification, and crystallization of soluble human decay accelerating factor expressed in E. coli."

BOGMGT/BOGDMS-695970-VI-P2006_000002-0720.DOC

REIVINDICACIONES

1. Un cristal de etanercept, en donde el cristal está en la forma de una barra o de una aguja.
2. Un cristal de etanercept como se describe en la reivindicación 1, en donde el cristal tiene una longitud máxima de entre 0.05 milímetros y 1.5 milímetros.
3. Un cristal de etanercept como se describe en la reivindicación 1, en donde el cristal tiene una longitud máxima de entre 0.05 milímetros y 0.3 milímetros.
4. Un cristal de etanercept como se describe en la reivindicación 1, en donde el cristal comprende una sal seleccionada del grupo que consiste de acetato de amonio, fosfato de amonio, sulfato de amonio, hidrogeno fosfato di-amonio, cloruro de litio, sulfato de litio, sulfato de magnesio, cloruro de potasio, citrato de potasio, fosfato de potasio,

acetato de sodio, cloruro de sodio, citrato de sodio, fosfato de sodio, y sulfato de sodio.

5. Un cristal de etanercept como se describe en la reivindicación 1, en donde el cristal comprende una sal seleccionada del grupo que consiste de acetato de amonio, fosfato de amonio, hidrogenfosfato diamonio, y cloruro de sodio.
6. Un método para fabricar un cristal de etanercept en la forma de una barra o de una aguja, en donde el método comprende combinar una solución de polipéptido de etanercept con un amortiguador de cristalización que comprende una sal.
7. El método de la reivindicación 6, en donde la combinación se coloca en equilibrio de vapor con un reservorio de amortiguador de cristalización.
8. El método de la reivindicación 6, en donde el amortiguador de cristalización tiene un pH entre 4.0 y 10.5.

9. El método de la reivindicación 6, en donde la sal se selecciona a partir del grupo que consiste de acetato de amonio, fosfato de amonio, sulfato de amonio, hidrogenfosfato diamonio, cloruro de litio, sulfato de litio, sulfato de magnesio, cloruro de potasio, citrato de potasio, fosfato de potasio, acetato de sodio, cloruro de sodio, citrato de sodio, fosfato de sodio, y sulfato de sodio.
10. El método de la reivindicación 6, en donde la concentración de sal en el amortiguador de cristalización está entre 0.04M y 1.2M.
11. El método de la reivindicación 6, en donde el amortiguador de cristalización incluye además 2-metil-2,4-pentanediol (MPD) o polietilenglicol (PEG).
12. El método de la reivindicación 6, que comprende además remover una porción del amortiguador de cristalización después de que se han formado los cristales.

13. El método de la reivindicación 12, en donde la porción del amortiguador de cristalización se remueve mediante centrifugación.
14. El método de la reivindicación 12, en donde los cristales se colocan en una solución que contiene un aditivo orgánico.
15. El método de la reivindicación 12, que comprende además la adición de un excipiente.
16. El método de la reivindicación 15 en donde el excipiente se selecciona a partir del grupo que consiste de sucrosa trehalosa, o sorbitol.
17. El método de la reivindicación 14 en donde el aditivo orgánico es etanol o isopropanol.
18. El método de la reivindicación 6, que comprende además secar los cristales que se han formado.
19. El método de la reivindicación 18 en donde los cristales se secan mediante exposición al aire, o

mediante exposición a un vacío, o mediante
exposición a gas nitrógeno.



(12) **United States Patent**
Osslund et al.

(10) **Patent No.:** **US 7,276,477 B2**
(45) **Date of Patent:** **Oct. 2, 2007**

- (54) **CRYSTALS OF ETANERCEPT AND METHODS OF MAKING THEREOF**
- (75) **Inventors:** **Timothy D. Osslund**, Camarillo, CA (US); **Christi L. Clogston**, Newbury Park, CA (US); **Shon Lee Crampton**, Los Angeles, CA (US); **Randal B. Bass**, Seattle, WA (US)
- (73) **Assignee:** **Amgen Inc.**, Thousand Oaks, CA (US)
- (*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 112 days.
- (21) **Appl. No.:** **10/901,735**
- (22) **Filed:** **Jul. 29, 2004**
- (65) **Prior Publication Data**
US 2005/0032183 A1 Feb. 10, 2005
- Related U.S. Application Data**
- (60) **Provisional application No. 60/491,827**, filed on Aug. 1, 2003.
- (51) **Int. Cl.**
A61K 38/00 (2006.01)
G01N 38/00 (2006.01)
- (52) **U.S. Cl.** **514/12; 436/4**
- (58) **Field of Classification Search** **514/12; 436/4**
- See application file for complete search history.

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Primary Examiner—Kathleen Kerr Bragdon
Assistant Examiner—Suzanne M. Noakes
(74) *Attorney, Agent, or Firm*—Kathleen Fowler; Suzanne Sprunger

(57) **ABSTRACT**

The present invention relates to crystalline etanercept and to methods of making crystalline etanercept; to pharmaceutical compositions comprising crystalline etanercept; and to therapeutic uses of such compositions.

29 Claims, 1 Drawing Sheet
(1 of 1 Drawing Sheet(s) Filed in Color)

-continued

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
210 215 220
Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Glu Pro Lys Ser Cys
225 230 235 240
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
245 250 255
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
260 265 270
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
275 280 285
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
290 295 300
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
305 310 315 320
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
325 330 335
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
340 345 350
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
355 360 365
Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser
370 375 380
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
385 390 395 400
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
405 410 415
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
420 425 430
Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
435 440 445
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
450 455 460
Pro Gly Lys
465

What is claimed is:

1. A crystal of etanercept in the form of a needle or a rod.
2. A crystal of etanercept as in claim 1, wherein the crystal is in the form of a rod.
3. A crystal of etanercept as in claim 1, wherein the crystal has a maximum length of between 0.5 millimeters and 1.5 millimeters.
4. A crystal of etanercept as in claim 1, wherein the crystal has a maximum length of between 0.05 millimeters and 0.3 millimeters.
5. A crystal of etanercept as in claim 1, wherein the crystal comprises a salt selected from the group consisting of ammonium acetate, ammonium phosphate, ammonium sulfate, di-ammonium hydrogen phosphate, lithium chloride, lithium sulfate, magnesium sulfate, potassium chloride, potassium citrate, potassium phosphate, sodium acetate, sodium chloride, sodium citrate, sodium phosphate, and sodium sulfate.
6. A crystal of etanercept as in claim 1, wherein the crystal comprises ammonium acetate, ammonium phosphate, di-ammonium hydrogen phosphate, and sodium chloride.

7. A method of making a crystal of etanercept, wherein the method comprises combining a solution of etanercept polypeptide with a crystallization buffer comprising a salt.

8. The method of claim 7, wherein the combination is placed in vapor equilibrium with a reservoir of crystallization buffer.

9. The method of claim 7, wherein the crystallization buffer has a pH between 4.0 and 10.5.

10. The method of claim 7, wherein the salt is selected from the group consisting of ammonium acetate, ammonium phosphate, ammonium sulfate, di-ammonium hydrogen phosphate, lithium chloride, lithium sulfate, magnesium sulfate, potassium chloride, potassium citrate, potassium phosphate, sodium acetate, sodium chloride, sodium citrate, sodium phosphate, and sodium sulfate.

11. The method of claim 7, wherein the concentration of salt in the crystallization buffer is between 0.04M and 1.2M.

12. The method of claim 7, wherein the crystallization buffer further includes 2-methyl-2,4-pentanediol (MPD) or polyethylene glycol (PEG).

13. The method of claim 7, further comprising removing at least a portion of the crystallization buffer after crystals have formed.

14. The method of claim 13 wherein the portion of crystallization buffer is removed by centrifugation.

15. The method of claim 13, wherein the crystals are placed in a solution containing an organic additive.

16. The method of claim 13, further comprising the addition of an excipient.

17. The method of claim 16 wherein the excipient is selected from the group consisting of sucrose, trehalose, or sorbitol.

18. The method of claim 15 wherein the organic additive is ethanol or isopropanol.

19. The method of claim 7, further comprising drying crystals that have formed.

20. The method of claim 19 wherein the crystals are dried by exposure to air, or by exposure to a vacuum, or by exposure to nitrogen gas.

21. An etanercept crystal produced by the method of claim 7.

22. A method of making a crystal of etanercept, wherein the method comprises combining a solution of etanercept polypeptide with co-solute means.

23. The method of claim 22 wherein the solution of etanercept polypeptide is further combined with crystallization buffering means.

24. A composition comprising an etanercept crystal of claim 1.

25. A method comprising administering to a subject an effective amount of the crystalline etanercept of claim 1.

26. The method of claim 25 wherein the subject has a condition characterized by excessive TNF-alpha levels.

27. The method of claim 26 wherein the administration of crystalline etanercept reduces levels of TNF-alpha in the serum or tissues of the subject.

28. The method of claim 26 wherein the subject has rheumatoid arthritis, psoriatic arthritis, psoriasis, or ankylosing spondylitis.

29. A composition comprising etanercept in crystalline form and an ingredient selected from the group consisting of: acidifying means, active ingredients, propellant means, aggregation inhibiting means, denaturant means, alkalizing means, anticaking means, antifoaming means, antioxidant means, formulation buffering means, chelating means, coating means, coloring means, complex-forming means, desiccating means, filtering means, flavoring means, moisture-retaining means, ointment means, plasticizing means, carrier means, preserving means, solubilizing means, stabilizing means, means for dissolving, adsorbing means, stiffening means, suppository means, viscosity-increasing means, sweetening means, tablet binding means, diluent means, tablet disintegrant means, lubricating means, tonicity modifying means, vehicle means, water-repelling means, and sustained-release means.

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Kenton L. Longenecker, Sarah M.
Garrard, Peter J. Sheffield and
Zygmunt S. Derewenda*

Department of Molecular Physiology and
Biological Physics, University of Virginia,
PO Box 800736, Charlottesville,
VA 22908-0736, USA

Correspondence e-mail: zsd4n@virginia.edu

Protein crystallization by rational mutagenesis of surface residues: Lys to Ala mutations promote crystallization of RhoGDI

Crystallization is a unique process that occurs at the expense of entropy, including the conformational entropy of surface residues, which become ordered in crystal lattices during formation of crystal contacts. It could therefore be argued that epitopes free of amino acids with high conformational entropy are more thermodynamically favorable for crystal formation. For a protein recalcitrant to crystallization, mutation of such surface amino acids to residues with no conformational entropy might lead to enhancement of crystallization. This paper reports the results of experiments with an important cytosolic regulator of GTPases, human RhoGDI, in which lysine residues were systematically mutated to alanines. Single and multiple mutations were introduced into two different variants of RhoGDI, NΔ23 and NΔ66, in which the first 23 and 66 residues, respectively, were removed by recombinant methods. In total, 13 single and multiple mutants were prepared and assessed for crystallization and all were shown to crystallize using the Hampton Research Crystal Screens I and II, in contrast to wild-type NΔ23 and NΔ66 RhoGDI which did not crystallize. Four crystal structures were solved (the triple mutants NΔ23:K135,138,141A and NΔ66:K135,138,141A, and two single mutants NΔ66:K113A and NΔ66:K141A) and in three cases the crystal contacts of the new lattices were found precisely at the sites of mutations. These results support the notion that it is, in principle, possible to rationally design mutations which systematically enhance proteins' ability to crystallize.

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PDB References: Δ23:
K135,138,141A mutant, 1fs;
Δ66 K135,138,141A;L196F
mutant, 1fs; Δ66 K113A
mutant, 1f0; Δ66 K141A
mutant, 1f3.

1. Introduction

Established protein crystallization methods rely on systematic screening for suitable conditions, including various precipitants, pH values, additives, temperature *etc.* In many cases, thousands of conditions are tested during screening; new crystallization robots are expected to set up over 100 000 samples daily (Stevens, 2000). However, the effectiveness of crystallization experiments is not directly proportional to the number of conditions tested and current attempts at global enhancement of structure-determination methodology (structural genomics) urgently require improvements in crystallization effectiveness.

It could be argued that the protein itself, rather than the precipitating agent, may be considered to be the most important variable in the screening process. Some proteins (*e.g.* lysozyme, insulin, hemoglobin) exhibit surface characteristics which lead to rapid crystallization under a variety of conditions, while other proteins have never been crystallized. Clearly, those proteins have surface properties that are not

favorable for the formation of crystal contacts. The possibility of using the protein as one of the variables in the process of screening for crystals has been suggested in the past. As early as 1972, it was proposed that if crystals of a target protein cannot be grown, homologous proteins from other species should be considered (Campbell *et al.*, 1972). In this way, these authors crystallized nearly every enzyme in the glycolytic pathway, albeit from different organisms. Although this approach is still often used, there is no way to predict if a protein from one species is more likely to crystallize than that from another. Another approach, which has so far enjoyed less popularity, is to mutate surface residues in search of a variant more susceptible to crystallization. One of the first examples of such protein engineering to promote crystallization was the work of Lawson and colleagues, who mutated human ferritin to generate crystal contacts analogous to those in the rat isoform (Lawson *et al.*, 1991). This was followed in 1992 by a seminal study which showed that even single-site amino-acid substitutions in thymidylate synthase substantially affected the crystallization of the protein (McElroy *et al.*, 1992). Since then, several examples of successful crystallization of mutants in lieu of wild-type proteins have been reported. During the studies of the HIV type 1 integrase, mutants in which hydrophobic residues were systematically replaced by lysines or alanines led to the identification of a single-site mutant, F185K, which gave crystals used in the successful structure analysis (Dyda *et al.*, 1994; Jenkins *et al.*, 1995). It has been reported that different mutants of T4 lysozyme can be crystallized in as many as 25 different forms (Zhang *et al.*, 1995).

The structural characterization of the *obese* protein leptin E100 (Zhang *et al.*, 1997) was possible because the W100→E mutation allowed the preparation of good crystals. Histidine ammonia lyase was successfully crystallized by exchange of a surface cysteine residue to an alanine (Schwede *et al.*, 1999). Very recently, D'Arcy and colleagues at Hoffman-La Roche used mutagenesis of surface residues in the DNA gyrase-B subunit to study the effect of surface substitutions on the ability of the protein to crystallize and obtained new crystal forms (D'Arcy *et al.*, 1999). In many cases, serendipity was a factor: the first crystals of the chaperone GroEL were obtained after errors were introduced inadvertently at the PCR stage (Horwich, 2000).

In spite of these results, the concept of crystal engineering has suffered from the lack of a rational approach: mutations were clearly recognized as a means to change proteins' solubility, but no rational protocol was ever designed to crystallize a new protein by surface mutagenesis. We hypothesized that mutagenesis of surface residues such as Lys and Glu to Ala or other smaller amino acids might systematically improve protein crystallization by creating epitopes more favorable for the formation of crystal contacts with negligible loss of conformational entropy. In this paper, we describe our initial testing of this approach on the human guanine nucleotide dissociation inhibitor RhoGDI. This physiologically important regulator of Rho-family GTPases has been previously shown to be recalcitrant to crystallization. RhoGDI is a single-chain protein made up of 204 residues, with a functional moiety restricted to the fragment 26–204 (Platko *et al.*, 1995). In the

absence of its target, Rho GTPase, residues 23–68 remain disordered (Gosser *et al.*, 1997; Keep *et al.*, 1997). Full-length protein and N-terminally truncated ($\Delta 23$ and $\Delta 66$) variants are all easily expressed in *Escherichia coli* using various fusion constructs (Sheffield *et al.*, 1999). A tryptic fragment (59–204) has been crystallized and its structure has been reported (Keep *et al.*, 1997) revealing an immunoglobulin-like fold; no successful crystallization of either the $\Delta 23$ or $\Delta 66$ variants has ever been described. The protein contains 14 lysines (10%) and 12 glutamates (8%) in the globular domain (67–210), making it a good candidate to test the Lys→Ala (K2A) concept (Fig. 1). We show that K2A mutations notably improve the success rate in crystallization of RhoGDI using commercially available Crystal Screen kits from Hampton Research. We also show that the integrity of crystal lattices in the

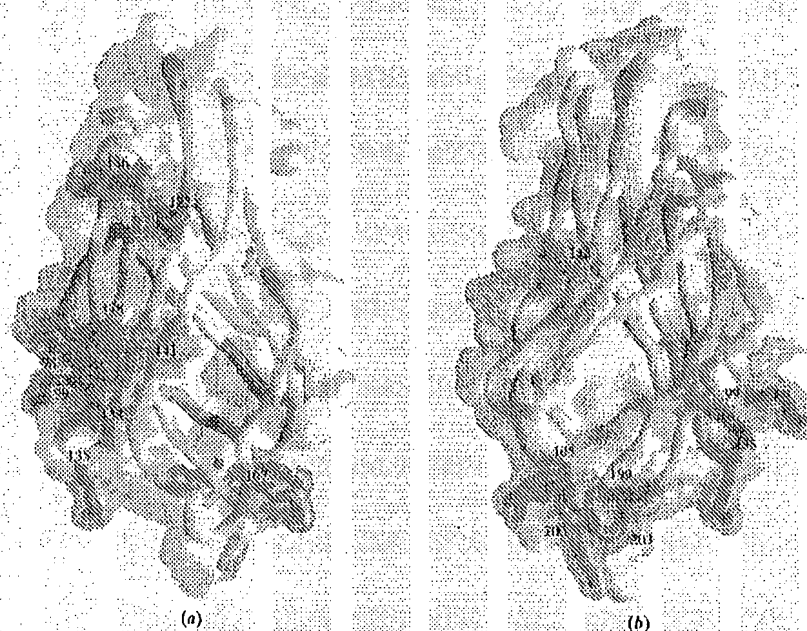


Figure 1. Overall structure of the ordered C-terminal domain of RhoGDI. Panels (a) and (b) show front and back perspectives of the β -sandwich topology of the immunoglobulin-like fold as viewed through a transparent rendering of the molecular surface. The 14 lysine residues in this domain are highlighted in blue.

Table 1
Primers used in the design of mutants

Mutation	Background	Primer sequence
K105A	Δ 23N	GCAGTCG1TTGTcCTGgcGGAGGGTGTGGAG-TACCGG
K113A	Δ 23N and Δ 66N	GGAGTACCGGATAgcATa1CTTCCGGG
K127A	Δ 23N	CCGAGAGATAGTGagCGGCATGgcGTACATCCAGC
K141A	Δ 23N and Δ 66N	GGCGTCAAAATcGAtgcGACTGACTACATGGTAGGC
K98,99,105A	Δ 23N and Δ 66N	CGGGCGACCTGGAGAGCTTCgcGgcG-CAaagcTTTGTGCTGgcGGAGGGT
K135,138,141A	Δ 23N and Δ 66N	CCAGCATACGTACAGGgcAGGCGTCgcGATTGACgc-GACTGACTACATGG
K98,99,105,113A	Δ 23N	Two-step mutagenesis using the primer for 113A and the template of 98,99,105
K98,99,105,141A	Δ 23N	Two-step mutagenesis using the primer for 141A and the template for 98,11,105

mutants is maintained predominantly by intermolecular contacts involving sites of mutations.

2. Materials and methods

2.1. RhoGDI plasmids and mutagenesis

Using pGEX2T1::RhoGDI wild-type plasmid as template DNA, new ATG start codons and *Nco*I sites were introduced into RhoGDI at positions 23 and 66 by the polymerase chain reaction. Two mutagenic primers (Δ 23N, 5'-GACCCAC-CATGGCCAACGTCGTGGTGACTGG; Δ 66N, 5'-GCAGACCCCATGGTCCCCAACGTCG-3') and a pGEX-specific primer (5'-CACCGTCATCACCGAAACGCGCGAGGC-3') were used to amplify the truncated RhoGDI genes. The subsequent products were digested with *Nco*I and *Eco*RI and subcloned into pGST-Parallel (Sheffield *et al.*, 1999) similarly digested. Positive clones, pGST::RhoGDI Δ 23N and pGST::RhoGDI Δ 66N, were verified by DNA sequencing. All mutations were introduced into genes coding for Δ 66 and Δ 23 RhoGDI in fusion with GST, with an rTEV proteinase cleavage site engineered for effective removal of the GST tag, as described elsewhere (Sheffield *et al.*, 1999). The QuikChange protocol has been used throughout, modified in our laboratory to reduce the cost. Primers were purchased from Life Technologies and used after dissolving in distilled water. Single and triple mutants described in our study were prepared using single mutagenic primers because of the proximity of the mutated sites; the triple mutants were prepared by mutagenesis of the plasmids carrying the triple mutation in a two-stage reaction. A complete list of primers and mutations is shown in Table 1.

2.2. Protein expression and purification

Expression of fusion proteins was carried out in 1–4 l of XL1 Blue cultures in LB medium incubated at 310 K until an OD₆₀₀ of 0.4–0.6. Expression was then induced by the addition of IPTG (final concentration 500 μ M). Cultures were grown for an additional 4 h at 310 K. Cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris-HCl pH 8.5, 300 mM NaCl; ~5 ml per gram of cell pellet) and lysed

using a French press. The soluble protein fraction was recovered by centrifugation. GST fusion proteins were isolated by binding to glutathione Sepharose columns (Pharmacia) pre-equilibrated with 100 ml of buffer A. The soluble protein fraction was passed through the column twice at 277 K and the columns were washed with 2 l of buffer A at 277 K. GST fusion protein was eluted in 20 ml of buffer containing 10 mM reduced glutathione pH 8.5. The GST tag was removed by treatment with 100 units of rTEV proteinase (Life Technologies), typically for 48 h at 283 K during dialysis against 2 l of buffer A, to remove the

glutathione before being reappplied to a glutathione Sepharose column to remove uncut protein as well as the contaminating GST tag. This procedure removed over 90% of the GST protein; the residual GST was removed by size-exclusion chromatography (Superdex 75). Residual rTEV was also removed by passing the purified protein through a mini-spin Ni²⁺ column (Qiagen). Proteins were analyzed by SDS-PAGE on 12% polyacrylamide gels in a Laemmli buffer system. Protein concentrations were measured using the Bradford assay (BioRad).

2.3. Crystallization

Proteins were crystallized at 293 K by vapor diffusion in VDX plates using Crystal Screens I and II (Hampton Research). Typically, 2 μ l protein solution was mixed with 2 μ l reservoir and suspended on a cover slip over a reservoir containing 0.5 ml precipitant. The concentration of the protein samples varied between 7 and 15 mg ml⁻¹. The Δ 23 K135,138,141A mutant crystallized in 12–14% PEG 3400, 5% 2-propanol and 100 mM HEPES pH 7.0. The Δ 66 triple mutant crystallized in 44–48% saturated ammonium sulfate and 100 mM MES pH 6.0. The Δ 66 single mutants crystallized similarly in 50% saturated ammonium sulfate, 200 mM sodium/potassium tartrate and 100 mM sodium citrate pH 5.6.

2.4. X-ray data collection

Single crystals of the mutants were transferred to a stabilizing solution containing the reservoir components and a cryoprotectant and were then flash-frozen by plunging into liquid nitrogen. The cryosolution for the Δ 23 triple mutant contained no 2-propanol but contained 30% PEG 400, whereas the cryoprotectant for the other crystals was 25–30% glycerol. X-ray data were collected on frozen crystals at beamline X11 at the EMBL Outstation Hamburg facility on a single-chip MAR Research CCD area detector and were processed with the *DENZO/SCALEPACK* set of programs (Otwinowski & Minor, 1997). Data for the Δ 66 triple mutant were similarly collected from a single crystal at beamline X9B of the NSLS (Brookhaven National Laboratory) and were processed as above. Intensities were converted to structure-

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Table 2

X-ray data statistics.

Values in parentheses are for the highest resolution shells, including data between $d_{\min}$ and 2.78, 2.07, 2.66 or 2.85 Å for each mutant, respectively.

	GDI-Δ23 K135,138,141A	GDI-Δ65 K135,138, 141A;L196F	GDI-Δ66 K113A	GDI-Δ66 K141A
Space group	<i>R</i> 3	<i>P</i> 3 ₂ 21	<i>R</i> 32	<i>R</i> 32
Unit-cell parameters (Å)				
<i>a</i> , <i>b</i>	125.2	76.3	128.5	129.6
<i>c</i>	82.7	88.6	164.3	164.8
$d_{\min}$ (Å)	2.7	2.0	2.6	2.8
No. of observations	49713	83504	73487	57299
No. unique	13241	20565	15110	13249
Completeness (%)	99.6	99.7	92.8	99.7
$I/\sigma(I)$	17.9 (3.7)	13.7 (4.8)	16.8 (2.7)	11.0 (1.9)
R_{sym} (%)	4.8 (26.7)	2.9 (30)	5.8 (33)	9.4 (68)
Beamline	EMBL X11	NSLS X9B	EMBL X11	EMBL X11

† $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I is the integrated intensity for a particular reflection.

Table 3

Structure refinement statistics.

	GDI-Δ23 K135,138,141A	GDI-Δ66 K135,138, 141A;L196F	GDI-Δ66 K113A	GDI-Δ66 K141A
Resolution range (Å)	20–2.7	20–2.0	20–2.6	20–2.8
<i>R</i> factor (%)	21.3	21.6	22.6	21.6
R_{free} † (%)	26.1	23.2	26.0	26.0
Reflections for R_{free}	1310	1050	1001	869
Protein atoms	2274	1106	2150	2155
Solvent molecules	40	199	56	36
Mean <i>B</i> factor (Å ²)	47.2	41.6	52.8	52.5
R.m.s.d. bonds (Å)	0.007	0.006	0.007	0.007
R.m.s.d. angles (°)	1.37	1.39	1.48	1.44

† R_{free} is calculated as the conventional crystallographic *R* factor, but using a randomly selected subset of reflections not incorporated into the refinement procedures.

factor magnitudes using the program *TRUNCATE* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994). Crystallographic details for all four sets are shown in Table 2.

2.5. Structure solution and refinement

Structures were solved by molecular replacement using the 2.5 Å resolution model of RhoGDI (chain A of PDB code 1rho) with the *AMoRe* program (Navaza, 1994). Molecular-replacement calculations on data from the Δ23 triple mutant yielded two independent solutions better than any others, which upon proper placement relative to one another yielded a correlation coefficient of 62 and an *R* factor of 37% for data in the resolution range 12–4.0 Å. Similar calculations on the Δ66 triple-mutant data (12–3.5 Å) yielded a single solution with a correlation coefficient of 49.8 and an *R* factor of 43%, whereas the values for the next best wrong solution were 21 and 53%, respectively. Molecular replacement on the Δ66 single-mutant data sets yielded only one clear individual solution, but after fixing this initial solution the second solution became apparent upon subsequent searches. The

Table 4

Hits obtained in crystallization screens.

CS1, Crystal Screen I; CS2, Crystal Screen II; the numbers correspond to the solution number in the Hampton Research Screens (components noted below)†. The bold numbers illustrate conditions which yielded crystals used in X-ray diffraction studies.

pGST-GDI	Δ23N	Crystals	Diffraction	CS1	CS2
K105A	Yes	—	—	4, 39 and 32	25, 32 and 14
K113A	Yes	—	—	32	15
K127A	Yes	—	—	16 and 17	23 and 25
K141A	Yes	—	—	4 and 32	14, 15 and 25
K98,99,105A	Yes	Yes	Yes	15	—
K135,138,141A	Yes	Yes	Yes	41	—
pGST-GDI Δ66N					
K113A	Yes	Yes	Yes	32	14
K141A	Yes	—	—	—	14
K98,99,105A	Yes	Yes	Yes	4, 6, 9, 15, 16, 17 and 33	20, 23, 25, 26, 32 and 42
K135,138,141A	Yes	Yes	Yes	—	32
pGST-GDI Δ23N 9899105					
K113A	Yes	—	—	39	25, 32
K141A	Yes	—	—	6, 15, 17	26

† In CS1: 4, 2.0 M ammonium sulfate (AS), 0.1 M Tris-HCl pH 8.5; 6, 30% (w/v) PEG 4000, 0.1 M Tris-HCl pH 8.5, 0.2 M MgCl₂ hexahydrate; 9, 30% PEG 4000, 0.1 M sodium citrate pH 5.6, 0.2 M ammonium acetate; 15, 30% PEG 8000, 0.1 M sodium cacodylate pH 6.5, 0.2 M AS; 16, 1.5 M lithium sulfate monohydrate, 0.1 M Na HEPES pH 7.5; 17, 30% PEG 4000, 0.1 M Tris-HCl pH 8.5, 0.2 M lithium sulfate monohydrate; 32, 2.0 M AS; 33, 4.0 M sodium formate; 39, 2% (v/v) PEG 4000, 2.0 M AS, 0.1 M Na HEPES pH 7.5; 41, 10% (v/v) propanol, 20% (w/v) PEG 4000, 0.1 M Na HEPES pH 7.5. In CS2: 14, 2.0 M AS, 0.1 M trisodium citrate dihydrate pH 5.6, 0.2 M potassium/sodium tartrate tetrahydrate; 15, lithium sulfate monohydrate, buffer as above, 0.5 M AS; 20, 1.6 M magnesium sulfate heptahydrate, 0.1 M MES pH 6.5; 23, 10% (v/v) dioxane, buffer as above, 1.6 M AS; 25, 1.8 M AS, 0.1 M MES pH 6.5; 26, 30% PEG MME 5000, buffer as above; 0.2 M AS; 32, 1.6 M AS, 0.1 M HEPES pH 7.5, 0.1 M NaCl; 42, 12% glycerol, 0.1 M Tris pH 8.5, 1.5 M AS.

combined solutions had a correlation coefficient of 49.7 and an *R* factor of 41.3% for data in the resolution range 10–4.0 Å.

The atomic models were refined against the structure-factor magnitudes using maximum-likelihood calculations implemented in *CNS* (Brunger *et al.*, 1998); visual inspection of electron-density maps and rebuilding were performed using *O* (Jones *et al.*, 1991). Solvent was identified automatically in difference maps and added manually for the lower resolution structures. The solvent structure for the Δ66 triple mutant was built using *ARP* in conjunction with *REFMAC*, both from the *CCP4* suite (Murshudov *et al.*, 1997; Perrakis *et al.*, 1999). The stereochemistry of the models was evaluated with *PROCHECK* (Laskowski *et al.*, 1993) and each was of acceptable quality. The results of the refinement are tabulated in Table 3. Figures were produced using *GRASP* (Nicholls *et al.*, 1991), *BOBSCRIPT* (Esnouf, 1997) and *MOLSCRIPT* (Kraulis, 1991).

3. Results and discussion

3.1. K2A mutations enhance crystallization of RhoGDI

Neither of the two N-terminally truncated variants of human RhoGDI (*i.e.* NΔ23 and NΔ66) crystallized from any of the Crystal Screen I and II solutions. Using the Δ23 construct of RhoGDI, we prepared four single mutants, two

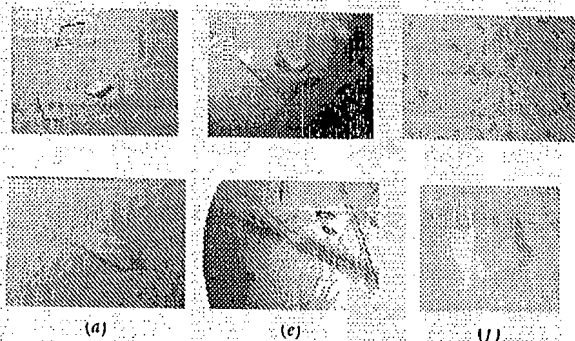


Figure 2

Microphotographs of selected crystals optimized from initial crystal hits. (a) $\Delta 23$ K135,138,141A triple mutant, CS1, 41. (b) $\Delta 66$ K113A single mutant, CS2, 14. (c) $\Delta 66$ K98,99,105A triple mutant, CS1, 33. (d) $\Delta 23$ K98,99,105A triple mutant, CS1, 15. (e) $\Delta 66$ K135,138,141A triple mutant, CS2, 32. (f) $\Delta 66$ K141A single mutant, CS2, 14.

triple mutants and two quadruple K2A mutants (Table 1). No special rationale was used in the selection of sites; in particular, triple mutants were designed based on the proximity of selected sites to allow mutagenesis using a single set of primers. All mutants gave 'hits' in either Crystal Screen I or II; five preparations (K105A, K127A, K141A and both quadruple mutants) gave three to five hits (Fig. 2; Table 4). It must be remembered that the $\Delta 23$ construct contains a disordered N-terminal fragment over 40 residues long. Thus, at least in the case of RhoGDI, surface engineering appears to outweigh the negative impact of partial disorder. The $\Delta 66$ construct, containing only the immunoglobulin-like domain, was used to generate two single mutants (K113A, K141A) and two triple mutants, both analogous to those tested in the $\Delta 23$ construct. Again, all samples gave hits in the Hampton screens, with the K98,99,105A triple mutant giving 13 hits in total (Table 4). In practically all cases crystallization occurred quickly, within hours or at most a few days of setting up the screens. Crystals

were obtained from a variety of solutions, but most hits were found in ammonium sulfate, with or without additives and with a range of buffers. Crystals also grew from PEG of higher molecular weights, lithium sulfate and magnesium sulfate. In one case, crystallization was observed in 4.0 M sodium formate solution.

Crystals of six of the mutants were assessed by X-ray diffraction using in-house facilities. Two, $\Delta 23$:K98,99,105A and $\Delta 66$:K98,99,105A, showed large unit cells which were found to be impractical for fast investigation by molecular-replacement procedures. The remaining four exhibited acceptable unit cells and were pursued further (Tables 2 and 3). The choice was somewhat arbitrary, since probably any of the initial 'hits' could have been optimized to yield large single crystals for X-ray diffraction. Two crystals selected for structure determination contain the triple mutation K135,138,141A, albeit in two different constructs with N-termini of various lengths. All four crystals were used to collect data to ~ 2.5 Å using synchrotron radiation. It was subsequently found that the $\Delta 66$:K135,138,141A mutant crystals diffracted significantly better when an additional mutation L196F, originally designed to probe the functionality of the hydrophobic pocket of RhoGDI in an unrelated study, was introduced. This did not change the crystallization habit (*i.e.* the crystals are isomorphous with those of $\Delta 66$: K135,138,141A), but increased the resolution to 2.0 Å. Thus, the discussion of crystal contacts in this form applies directly to the triple mutant.

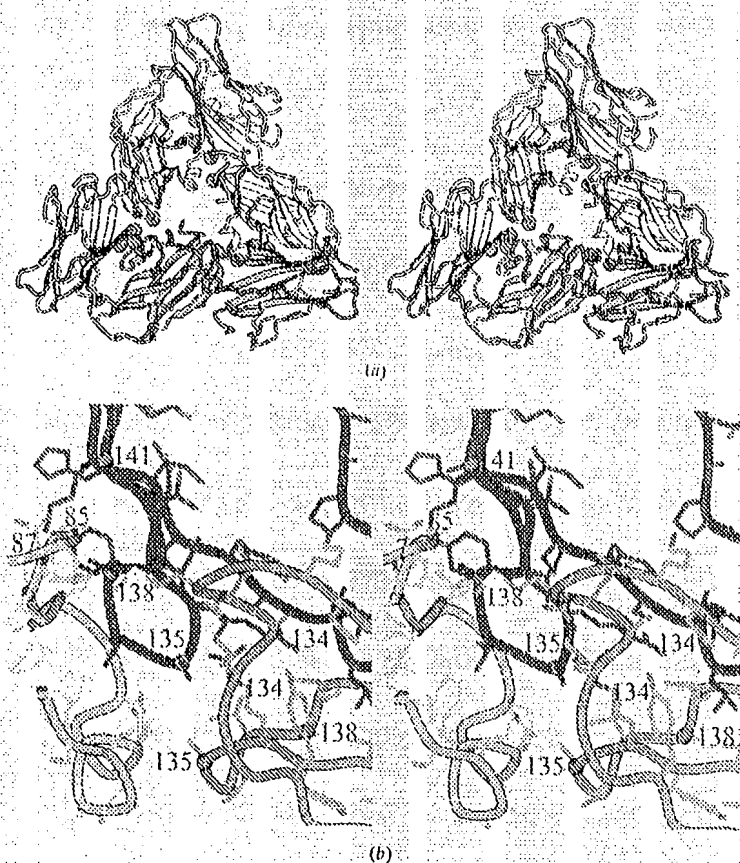


Figure 3

Stereoview of the molecular packing in crystals of the $\Delta 23$ triple mutant. (a) Several molecules are shown from a view looking down the crystallographic threefold axis. The location of K2A point mutations are highlighted with magenta balls. (b) A close-up view reveals molecular interactions between molecule A (grey) and molecule B (green) of the asymmetric unit as well as another molecule (orange) related by crystallographic symmetry.

3.2. The crystal structure of the $\Delta 23$ K135,138,141A mutant

The crystal structure of the $\Delta 23$:K135,138,141A triple mutant was solved by molecular replacement and refined at 2.7 Å resolution to a conventional R factor of 21.3% (R_{free} of 26.1%). This crystal form (space group $R3$) contains two protein molecules (denoted A and B) related by non-crystallographic twofold symmetry. The solvent content is fairly high, reflected in a Matthews coefficient of $3.1 \text{ Å}^3 \text{ Da}^{-1}$. The non-crystallographic dimer is formed by a tightly packed symmetric interface that is centered upon Arg134 and has

numerous van der Waals interactions, including the Ala135 introduced in place of a lysine (Fig. 3a). Adjacent to this interface, we find another close contact between the A molecule and another B molecule, mediated by the epitope containing mutated sites 138 and 141. The nature of this interface is decidedly hydrophobic, with Pro85 of molecule B lodged between the C^β methyl groups of Ala138 and Ala141. The contact is further stabilized by the antiparallel association of two short stretches of main chain, so that two strong main-chain-main-chain hydrogen bonds are made: B87 CO to NH of A138 (2.97 Å) and A136 CO to NH of B89 (2.96 Å). It is quite clear that this close interface, involving primarily main-chain atoms, could not form with the wild-type lysines in positions 135, 138 and 141 (Fig. 3b).

In addition to the contacts involving the mutated epitope, molecule B makes an additional extensive contact with an adjacent pair of symmetry-related A molecules mediated by the N-terminal fragment encompassing residues 56–65. This fragment, not present in the $\Delta 66$ construct, wedges between the two A molecules in such a way that it forms an additional strand along one of the β -sheets in RhoGDI. This interesting and extensive contact is obviously insufficient to generate crystals of the wild-type $\Delta 23$ RhoGDI; it appears that the above-described contacts involving the mutated 135, 138 and 141 sites play a critical role.

3.3. The structure of the $\Delta 66$ K135,138,141A mutant

In spite of the same set of surface mutations as the above sample, the shorter $\Delta 66$ variant forms crystals exhibiting different symmetry, i.e. $P3_221$. Although considerations based on the Matthews coefficient (V_M of $4.6 \text{ Å}^3 \text{ Da}^{-1}$, assuming one molecule) initially indicated that two molecules may occupy the asymmetric unit in these crystals, subsequent molecular-replacement calculations persistently gave only one solution. This solution was subjected to refinement using data to 2.0 Å resolution and the process converged well with a conventional R factor of 21.6% (R_{free} = 23.2%). The packing diagram (Fig. 4a) shows how an elongated molecule of RhoGDI forms a stable crystal lattice mediated through crystal contacts involving residues at two opposite ends.

The loop responsible for much of the crystal-forming interface is again precisely

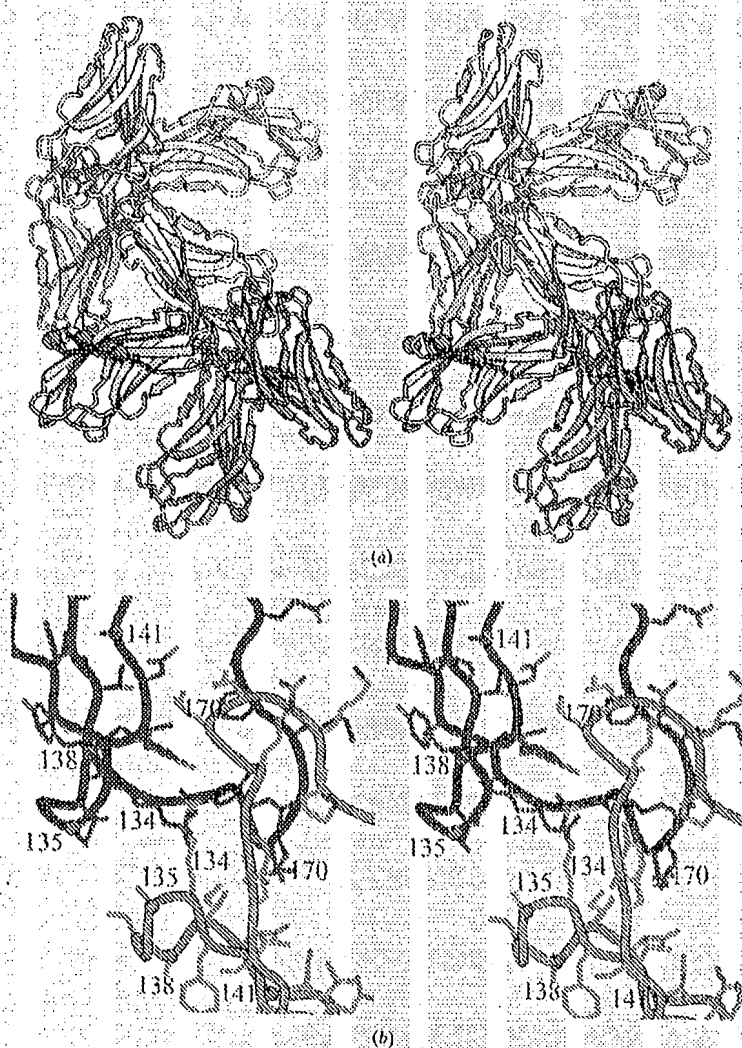


Figure 4
Stereoview of the molecular packing in crystals of the $\Delta 66$ triple mutant. (a) Several molecules are shown from a view looking down the crystallographic threefold screw axis. The location of K2A point mutations are highlighted with magenta balls. (b) A close-up view reveals molecular interactions between two molecules related by crystallographic symmetry. This view is reminiscent of the packing of non-crystallographic dimers in the other crystal forms. For clarity, this view does not show the packing of two other crystallographically related molecules, which are also in close proximity and are apparent in Fig. 4(a).

the loop containing the triple mutation K135,138,141A (Fig. 4b). This interface brings into proximity two symmetry-related pairs of molecules, so that two molecules contribute the 135,138,141 loop while the other two are involved via the topologically opposite end. The resulting extensive interface is populated with small mostly hydrophobic residues with low conformational entropy, e.g. Val137, Val67, Ala153, Pro151, Ile122 and notably Ala135 and Ala138. There are few direct interactions and only three hydrogen bonds, all involving main-chain atoms: 139 CO to 67 NH (3.2 Å), 120 CO to 174 NH (2.8 Å) and 167 CO to 167 NH (3.2 Å) from another molecule. Lack of a larger number of direct bonds confers a character of non-specificity on this interface. The void spaces in this interface are filled with ordered water molecules forming local networks of hydrogen bonds.

3.4. The structures of the $\Delta 66$ K113A and K141A single-site mutants

Both single mutants K113A and K141A crystallize in the same crystal form (space group $R32$) and like the previous crystals forms show a relatively high solvent content, with a Matthews coefficient of $4.1 \text{ \AA}^3 \text{ Da}^{-1}$. The asymmetric unit

contains a dimer which is conspicuously similar to the dimer found in the $\Delta 23$ K135,138,141A mutant in that the two molecules insert the hydrophobic residues of the 169–170 loop into the hydrophobic cavity of the adjacent molecule (Fig. 5a). The rest of the dimer is largely free of crystal contacts, with the exception of the surface around Ala113, the site of mutation in one of the mutants. The limited contact in this area involves several residues of mixed character, involving Ala113, Val78 and a few neighboring residues from the A molecule packed against Pro85, Glu87 and Val78 in the symmetry-related B molecule (Fig. 5b). There are no immediately obvious direct hydrogen bonds at this interface, but water does fill the void spaces. In the K141A mutant, the contact retains its integrity, even though Lys113 now fills some space at the interface. Interestingly, the mutation site is distant from any crystal contacts and it is difficult to rationalize why the K141A mutant yielded this crystal form whereas other preparations of $\Delta 66$ truncation mutants did not.

3.5. Overview of the structural data

The choice of Lys $\rightarrow$ Ala (K2A) mutations in this pilot study was dictated by many factors. Lysines are predominantly located on the surface, with 68% exposed, 26% partly exposed and only 6% buried (Baud & Karlin, 1999). In the case of RhoGDI, all of the lysines are either completely or partially exposed on the surface. On average, Lys is the most solvent-exposed residue in proteins. Statistically, these amino acids constitute 5.8% of the total amino-acid content in proteins, ranging from zero in rare and small acidic proteins to over 10% in some microtubule-associated proteins. It is important in this context to realise that even in a protein with average lysine content (5.8%) these amino acids constitute a large fraction of the surface area because nearly all are solvent accessible. Thus, even in a protein with an unknown fold, lysines provide markers for identifying a solvent-accessible site with a better than 90% success rate. Intuitively, we assume that the high conformational entropy of solvent-exposed lysines, $\sim 8 \text{ kJ mol}^{-1}$ (Avbelj & Fele, 1998), is likely to impair the formation of protein-protein contacts. This is supported by a recent study which shows that lysines constitute only 5.4% of interface surface in oligomeric proteins, compared with 14.9% of the total solvent-accessible surface (Conte *et al.*, 1999).

We hypothesized that mutating Lys to Ala may be effective in enhancing crystallizability, as alanine is evenly distributed

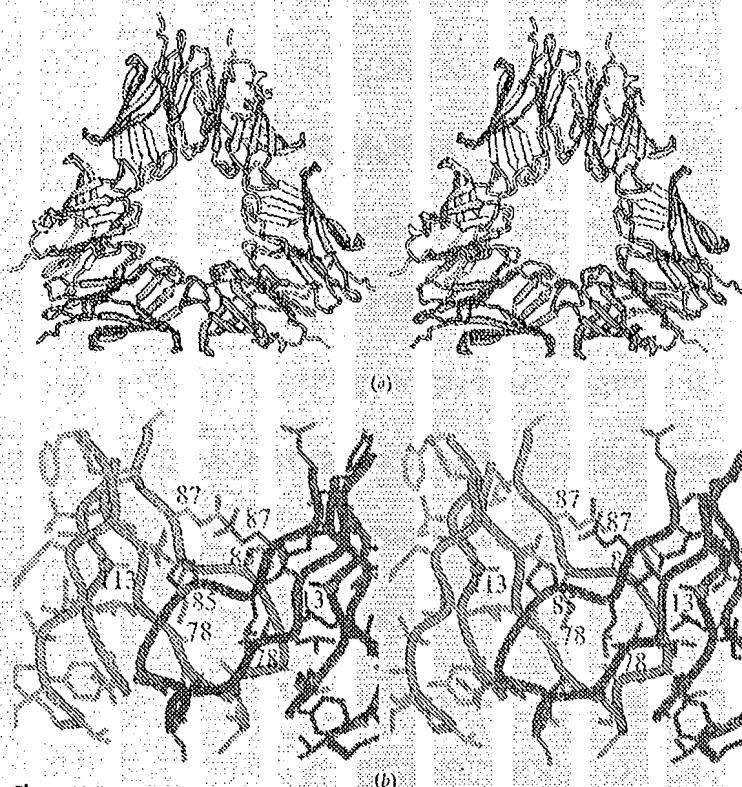


Figure 5

Stereoview of the molecular packing in crystals of the $\Delta 66$ K113A single mutant. (a) An overall view is shown looking down the crystallographic threefold axis. The location of residue 113 is highlighted with magenta balls. (b) A close-up view reveals molecular interactions between molecule A (grey) and molecule B (green) of the asymmetric unit.

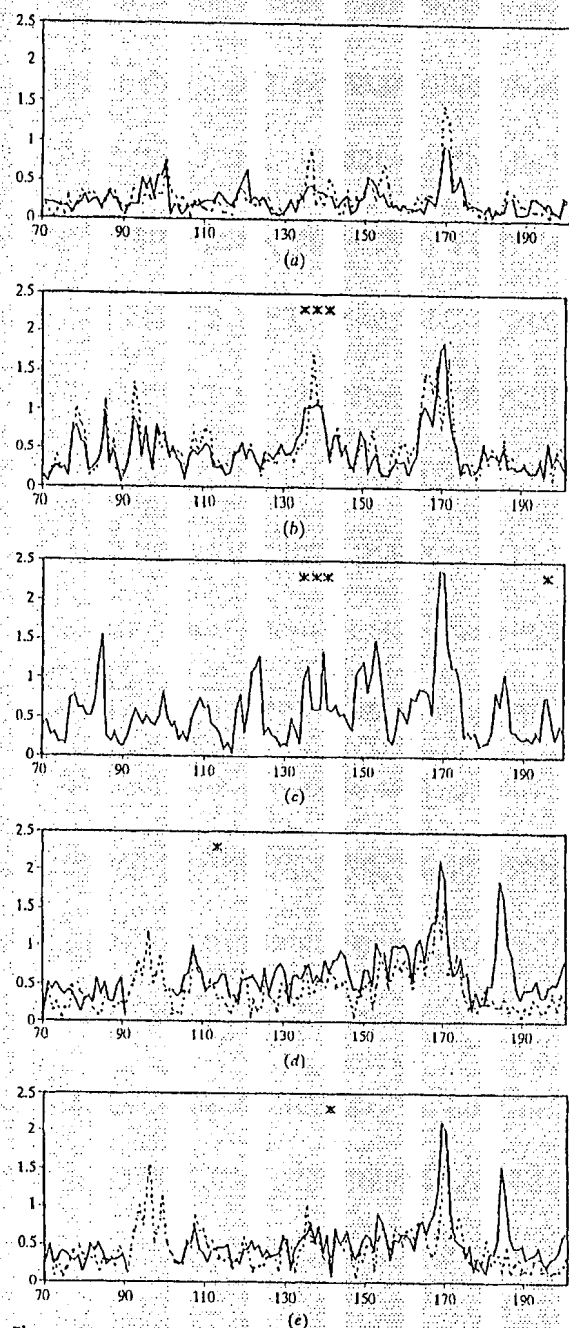


Figure 6
Comparison of the differences between structures of RhoGDI. Plots are shown for the distances (Å) between the C^α positions of aligned RhoGDI structures as a function of residue position. Positions of point mutations are highlighted with an asterisk. (a) Difference plots are shown for the three molecules in the asymmetric unit of the native structure (PDB code 1rho): molecule A compared with molecule B (solid) or molecule C (dashed) of the asymmetric unit. (b)–(e) Difference plots are shown for comparisons between molecule A of 1rho and the mutant structures reported in this manuscript, using solid lines for molecule A and dashed lines for molecule B where appropriate. The mutants are as follows: (b) $\Delta 23$ triple mutant, (c) $\Delta 66$ triple mutant, (d) $\Delta 66$ K113A single mutant and (e) $\Delta 66$ K141A single mutant.

in the interior, on the surface and at the interface in oligomeric proteins (Conte *et al.*, 1999) and has no conformational entropy. With the exception of the K141A mutant, the structural data strongly supports our hypothesis that reduction of surface conformational entropy by mutagenesis can lead to the creation of epitopes thermodynamically suitable for the formation of crystal lattices. In the two triple-mutant structures, the sites of mutation are clearly involved in the crystal contacts and seem to be directly responsible for the crystallization habit of the protein. The crystal contacts involve numerous interactions between main-chain atoms, including hydrogen bonds between main-chain carbonyls and amides. It is not clear to what extent the K113A mutation was helpful in promoting the R32 crystal form. Although residue 113 is located at an intermolecular crystal contact, the remotely positioned K141A mutation leads to a similar packing arrangement with minimal changes to accommodate the lysine at position 113. While all K2A mutants proved more susceptible to crystallization efforts, we infer from the current data that multiple mutations are perhaps more likely to generate useful contiguous surface epitopes allowing the preparation of crystals in a variety of lattice arrangements.

The structures solved in our study indicate that K2A mutations did not cause any significant structural perturbations in RhoGDI. We have analyzed the structures of all mutants by overlapping them using a least-squares algorithm and calculating plots of C^α differences compared with the original search model (Fig. 6). In general terms, we note no significant conformational changes arising from mutations, even in the mutants containing triple K2A substitutions located in close proximity along the polypeptide chain. Some deviations are observed in the C^α plots, but they are smaller than the differences observed for the 170 loop which is also involved in crystal contacts. There are only minor conformational changes in the side chains surrounding the mutation sites and they appear to be caused by crystal packing interactions rather than the mutations themselves.

A comparison of the C^α coordinates in the two molecules of the triple $\Delta 23$ mutants with the search model from the PDB shows that the mean r.m.s. for the C^α positions is about 0.7 Å, consistent with the limited resolution of both X-ray studies. The largest deviation of nearly 2 Å is found in the flexible loop that contains residue 170, a pattern that we also observe in other structures. The fragment containing the mutated sites shows slightly higher deviation than average and the two molecules differ somewhat in the extent of changes. Since this site mediates crystal contacts, it is difficult to judge if the observed differences are a consequence of mutagenesis or of the crystal contact. A comparison of C^α coordinates of the 2.0 Å resolution structure of the triple $\Delta 66$ mutants with the search model reveals an overall r.m.s. of approximately 0.75 Å, with few C^α atoms differing by more than 1.0 Å. Many of the differences are likely to stem from the relative inaccuracy of the 2.5 Å resolution search model. The C^α atoms of the mutated sites are within this range, while the largest discrepancy is again observed for the loop that contains residue 170. The plots of C^α differences for the two single-site mutants

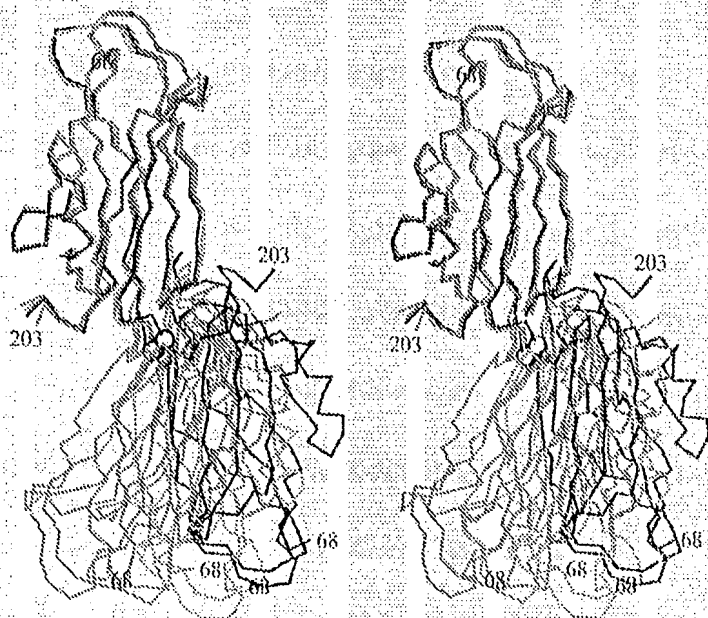


Figure 7
Similarity of the molecular interactions between various crystal forms of RhoGDI. A stereoview highlights the propensity of RhoGDI to form dimers, an arrangement that is evident in all crystal forms. Overlaying the structures (C traces) of one of the molecules from the dimer shows the relative placement of the other molecule. The crystals represented are of the wild-type (black), the $\Delta 23$ triple mutant (red), the $\Delta 66$ quadruple mutant (cyan) and the $\Delta 66$ K113A single mutant (green). The location of residue 170 is highlighted in the aligned molecules with yellow spheres.

reveal no major changes when compared with the search model, again with the notable exception of the 170 loop, which deviates by over 2 Å.

Parenthetically, we note that the RhoGDI molecules have a propensity to form non-crystallographic dimers in which the hydrophobic entrances to the central cavity face each other and in all crystal lattices a contact of this nature is evident (Fig. 7). The 170 loop, whose apex sequence is GMLA, is invariably in close contact with the residues lining the entrance in the adjacent molecule and its conformation is altered as the mutual disposition of the two RhoGDI molecules varies from dimer to dimer. This explains the observed conformational flexibility of the 170 loop seen in the different crystal lattices.

4. Conclusions

Our results strongly suggest that, at least in the model system of RhoGDI, K2A mutagenesis led to improved crystallization results. Out of the 13 various mutants, all gave hits and several gave diffraction-grade crystals in the first screens. Furthermore, the structures of four mutants reveal that crystal contacts are in fact mediated by epitopes involving the mutated sites. These results strongly suggest a causal rela-

tionship between the Lys→Ala mutations and enhanced crystallization of the RhoGDI mutants compared with the wild type.

To date, site-directed mutagenesis has not been used widely to enhance crystallization, probably owing to the lack of established protocols that would provide guidance to the type of mutation that is required. The approach we propose is based on a hypothesis that residues with high conformational entropy may impede crystallization. Other factors may clearly contribute to the enhanced crystallizability of K2A mutants (e.g. removal of potential steric hindrance, alteration of electrostatic charge, the hydration shell etc.), but whatever the rationale, the important outcome of our study is the demonstration of a causal relationship between K2A mutations and crystallization. To establish whether that is indeed the case, we obviously need to test other types of mutations (involving, for example, Glu and Gln) and other protein systems. This work is under way.

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Biological activity, membrane-targeting modification, and crystallization of soluble human decay accelerating factor expressed in *E. coli*

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Biological activity, membrane-targeting modification, and crystallization of soluble human decay accelerating factor expressed in *E. coli*

JENNIFER WHITE,¹ PETRA LUKACIK,² DIRK ESSER,¹ MICHAEL STEWARD,¹ NAOMI GIDDINGS,¹ JEREMY R. BRIGHT,¹ SARAH J. FRITCHLEY,¹ B. PAUL MORGAN,³ SUSAN M. LEA,² GEOFFREY P. SMITH,⁴ AND RICHARD A.G. SMITH¹

¹Adprotech, Ltd., Saffron Walden, Essex CB10 1XL, United Kingdom

²Laboratory of Molecular Biophysics, Biochemistry Department, Oxford University, Oxford OX1 3QU, United Kingdom

³Department of Medical Biochemistry and Immunology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, United Kingdom

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Abstract

Decay-accelerating factor (DAF, CD55) is a glycosphosphatidyl inositol-anchored glycoprotein that regulates the activity of C3 and C5 convertases. In addition to understanding the mechanism of complement inhibition by DAF through structural studies, there is also an interest in the possible therapeutic potential of the molecule. In this report we describe the cloning, expression in *Escherichia coli*, isolation and membrane-targeting modification of the four short consensus repeat domains of soluble human DAF with an additional C-terminal cysteine residue to permit site-specific modification. The purified refolded recombinant protein was active against both classical and alternative pathway assays of complement activation and had similar biological activity to soluble human DAF expressed in *Pichia pastoris*. Modification with a membrane-localizing peptide restored cell binding and gave a large increase in antihemolytic potency. These data suggested that the recombinant DAF was correctly folded and suitable for structural studies as well as being the basis for a DAF-derived therapeutic. Crystals of the *E. coli*-derived protein were obtained and diffracted to 2.2 Å, thus permitting the first detailed X-ray crystallography studies on a functionally active human complement regulator protein with direct therapeutic potential.

Keywords: complement; decay-acceleration; membrane anchor; crystallography

Reprint requests to: Richard A.G. Smith, Adprotech Ltd., Chesterford Research Park, Little Chesterford, Saffron Walden, Essex CB10 1XL, UK; e-mail: r.a.smith@adpro.co.uk; fax: +44 (0) 1799 532543.

⁴Present address: Solexa Ltd., Saffron Walden, Essex CB10 1XL, UK. **Abbreviations:** CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GPI, glycosphosphatidyl inositol; PpDAF, human DAF1-4 expressed in *Pichia pastoris*; N glycosylated and with an oligohistidine tag; EcDAF, nonglycosylated human DAF 1-4 expressed in *Escherichia coli*; nDAF, human native glycosylated (GPI-anchored) DAF from erythrocytes; EcDAF-MP, soluble *E. coli* human DAF linked through a C-terminal cysteine to the myristoylated peptide APT542; PCR, polymerase chain reaction; SCR, short consensus repeat; TCEP, *Tris*-(2-carboxyethyl) phosphine.

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DAF is a GPI-anchored glycoprotein that regulates complement activation, preventing the destruction of host tissue. It is widely distributed in tissue, especially upon endothelial cells (Nishikawa et al. 1998), but significant variations in expression have been noted within specific organs such as the kidney (Spiller et al. 1999) and in skin (Sayama et al. 1991). DAF plays a role in local control of complement activation, for example, on melanocytes (Vennekler et al. 1998) and on the endometrium (Young et al. 2002).

DAF is a member of the regulators of complement activation (RCA) gene family of proteins (Hourcade et al. 1989), which inhibit the C3 and C5 convertases, the central

amplification enzymes of the complement cascade. The RCA family includes the fluid phase regulators Factor H and C4b binding protein; and the membrane bound proteins DAF, membrane cofactor protein (MCP or CD46), and complement receptor type 1 (CR1 or CD35). Membrane-associated DAF prevents the assembly of the C3 convertases C4b2a and C3bBb by dissociating C2a and Bb from the complexes (Medof et al. 1984; Fujita et al. 1987) and acts only on convertase complexes generated on the same membrane and not extrinsically on convertases assembled on target cells (Medof et al. 1984). DAF is normally expressed as a glycosylated 70-kDa protein, although an 82-kDa form has been described (Hensel et al. 2001). The functional sites of DAF are contained within four short consensus repeats (SCRs, also known as complement control protein motifs—CCPs—or sushi domains). DAF is therefore structurally related to other complement regulatory proteins such as CR1/CD35 and MCP/CD46, which are also composed of SCR domains; CR1 allotypes have up to 44 such repeats, and MCP, like DAF, has 4. It has been shown that classical and alternative pathway regulatory activity of DAF is associated with SCRs 2 and 3 and SCRs 2, 3, and 4, respectively (Brodbeck et al. 1996). DAF has an N-linked glycan between SCRs 1 and 2, but it has been shown (Brodbeck et al. 2000) that the N glycan is not required for the complement regulatory function of DAF. A membrane-proximal O-linked polysaccharide rich region (S/T region) separates the SCRs from the membrane to which the protein is tethered by a GPI anchor (Medof et al. 1986). It has been suggested that the S/T region may be a functional spacer (Coyne et al. 1992), because deletion mutants (DAFΔS/T), although expressed normally, were nonfunctional. Forms of DAF with the GPI anchor replaced by an encoded transmembrane domain were, however, shown to be active (Lublin and Coyne 1991). Fodor et al. (1995) transfected a murine fibroblast cell line with CD59/DAF chimeric proteins, and found that, unlike CD59, DAF does not have a requirement to be proximal to the membrane. The outline structures of the various forms of DAF used in this study are shown in Figure 1.

Moran et al. (1992) engineered soluble and secretory forms of DAF lacking GPI anchors. These anchorless forms of DAF were 50-fold less active than GPI-anchored DAF extracted from CHO cells at inhibiting complement activation on the cell surface *in vitro*, and they inhibited fluid phase activation where the GPI-anchored DAF did not. The soluble form of DAF was found to inhibit the reversed passive Arthus reaction in guinea pigs.

Encouraged by this and by more recent studies (Harris et al. 2002) on the biological activity of soluble forms of DAF, our aim was to express DAF in *Escherichia coli* and refold it to produce an active protein for therapeutic applications and structural studies. We have described elsewhere a general strategy for restoring membrane-binding function in

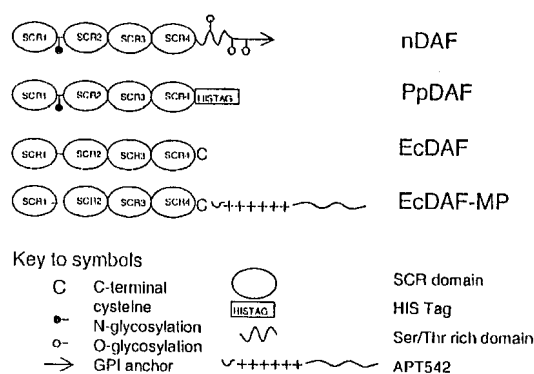


Figure 1. Summary of the domain structures and modifications made to the DAF forms used in this study. For definitions of the forms, see Abbreviations. Note that the exact composition of the glycoform carbohydrate differs between nDAF and PpDAF.

recombinant soluble complement regulatory proteins by modification with synthetic peptide derivatives (Smith and Smith 2001; Fraser et al. 2002, 2003; Smith 2002). Such a modification strategy has the advantage that it enables the large-scale production of soluble proteins derived from the extracellular domains of membrane-bound proteins and does not require extraction of such proteins from host cell membranes.

This approach has been shown to permit novel therapeutic applications of complement regulators in animal models of disease (Dong et al. 1999; Linton et al. 2000), and one modified agent derived from CR1 has progressed to human clinical studies (Smith 2002). The purpose of the present studies was therefore to establish expression and refolding conditions for large-scale production of DAF derivatives with therapeutic potential and utility for structural studies. Large-scale production is facilitated by utilizing an *E. coli* expression system which, in this case, has an added benefit of not introducing glycosylation into the DAF molecule. Glycosylation can complicate crystallization and NMR studies by introducing heterogeneity, and is itself not required for the function of DAF (Brodbeck et al. 2000; Kuttner-Kondo et al. 2001).

Structural studies on SCR-containing proteins have been reported previously (Norman et al. 1991; Barlow et al. 1992; Wiles et al. 1997), but only limited information is available on proteins containing more than two such domains. The pathogen binding domains of DAF (SCRs 3 and 4) have been expressed in the yeast *Pichia pastoris* and their structure determined to 1.7 Å (Williams et al. 2003). Additionally, Uhrinova et al. (2003) have solved the solution structure of DAF modules 2 and 3 that are necessary and sufficient to accelerate decay of the classical pathway convertase. However, prior to the present work no high-resolution structure of a multi-SCR human protein with intact

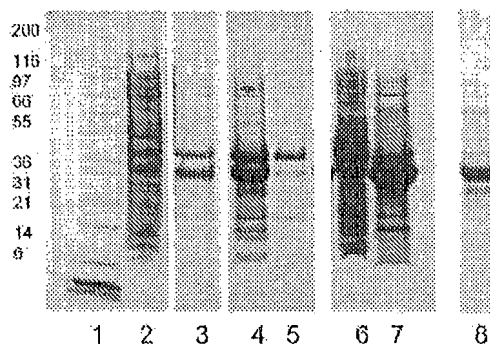


Figure 2. Expression and refolding of EcDAF. Reducing 4%–12% PAGE gels. Lane 1, MW markers; lane 2, homogenized cell pellet; lane 3, pellet wash (PBS/Tween-80); lane 4, supernatant after solubilization; lane 5, pellet after solubilization; lane 6, pellet after dialysis against 6 M urea, 10 mM HCl; lane 7, supernatant after dialysis; lane 8, soluble product post-refold.

complement (classical and alternative) regulatory function had been described.

We demonstrate here that it is possible to produce recombinant soluble DAF from *E. coli*-derived inclusion bodies, that the material produced is active in decay-acceleration and hemolytic assays of both the classical and alternative pathways, that it can be modified in a site-specific fashion to restore membrane binding function with an associated increase in potency, and that it can be crystallized.

Results

DNA encoding a DAF protein comprising the four extracellular SCR domains with a C-terminal cysteine (Fig. 1) was cloned into a pET-based vector (see Materials and Methods) and expressed in *E. coli*. The C-terminal cysteine was added to permit site-specific terminal localization of a membrane-targeting peptide. The product was isolated and solubilized from inclusion body pellets. Using high-pH refolding conditions similar to those previously employed for multi-SCR constructs derived from CR1 (Dodd et al. 1995; Mossakowska et al. 1999), we obtained a soluble protein that could be isolated in ~80% purity (as assessed by non-reducing SDS-PAGE [Fig. 2, lane 8]) using simple fractional precipitation and gel filtration methods.

For crystallization, detailed assay studies, and for modification, the EcDAF preparation was further purified by gel filtration yielding fractions with different mobilities on nonreducing SDS-PAGE gels. Only one fraction had the same mobility as PpDAF (data not shown), and this was selected for use in crystallization trials and most assays, because PpDAF is known to be correctly folded from studies with conformationally sensitive antibodies and from NMR studies (Powell et al. 1997). The results were cor-

roborated by MALDI-TOF mass spectrometry (MS), which confirmed that the isolated species had the correct molecular mass as predicted from the amino acid sequence.

To confer membrane binding, EcDAF was site-specifically modified with a synthetic analog of a "myristoyl-electrostatic switch" peptide, APT542 (Smith and Smith 2001).

This resulted in approximately 95% conversion to a singly modified species as observed by a gel shift (Fig. 3) confirmed by MALDI-TOF MS.

Decay acceleration assays in which the C3 convertases of each pathway were assembled from purified components (see Materials and Methods) were used to check this key activity in EcDAF. Figure 4 shows that the concentration of EcDAF required to reduce C3a production by 50% was approximately 30 nM in classical pathway (C1s triggered) activation and <10 nM for the alternative pathway (factor D triggered). This potency was comparable to or better than that of reference standards (e.g., SCR1-3 of CR1 which has an IC_{50} value of ~100 nM against the classical pathway and ~20 nM against the alternative pathway—data not shown). Under the conditions of these fluid-phase assays detergent-solubilized nDAF was only weakly active. Purified DAF derivatives were also assayed for activity in hemolytic assays using the classical and alternative pathways of complement activation. In a classical pathway hemolytic assay the EcDAF preparation was shown to be of comparable potency to soluble PpDAF (Fig. 5A).

Figure 5B compares purified EcDAF, GPI-anchored nDAF, and EcDAF-MP in a classical pathway antihemolytic assay. The IC_{50} of EcDAF-MP was ~0.2 nM in this assay, approximately 100-fold more potent than the unmodified protein and significantly more active than detergent-solubilized nDAF. The detergent CHAPS was required

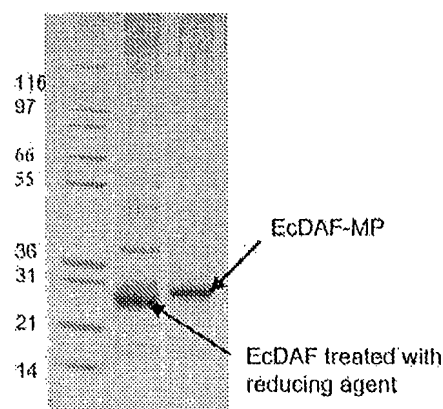


Figure 3. Conjugation of EcDAF with the membrane-localizing peptide MP (APT542). Nonreducing 4%–12% PAGE gels. Lane 1, MW markers; lane 2, EcDAF reduced with TCEP; lane 3, EcDAF reduced with TCEP and modified with MP.

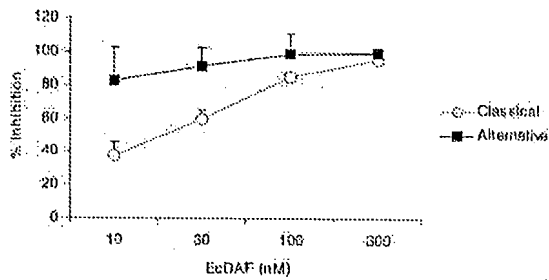


Figure 4. Decay-accelerating activity of EcDAF in fluid-phase assays: Inhibition of C3a release from the classical pathway (open circles) and alternative pathway (filled squares) convertases. Means $\pm$ SD of two separate experiments.

to ensure the solubility of nDAF, and the possibility cannot be ruled out that CHAPS inhibits incorporation of GPI-anchored proteins into cell membranes and thus causes the potency of nDAF in this assay to be underestimated.

The effect of CHAPS on EcDAF-MP activity was investigated and found to be negligible except at a concentration of <0.5 nM where a slight potentiating effect was observed (data not shown). In an alternative pathway hemolytic assay using a 1 in 8 final dilution of serum, the IC_{50} of EcDAF was approximately 800 nM (Fig. 5C) and using 1 in 16 final serum dilution it was lowered to 30–40 nM (Fig. 5D). Under

both alternative pathway assay conditions, there was no significant difference in potency between modified and unmodified forms of EcDAF. When this assay was repeated in a “wash” format (i.e., with the target erythrocytes exposed to agents in the absence of serum, followed by buffer washing and exposure to diluted serum), EcDAF-MP was not able to protect the target cells (data not shown). However, it was active if present in the assay mixture continuously (see Fig. 5C,D).

Binding of EcDAF-MP to cells could be demonstrated directly under other conditions. Figure 6, A and B, compares Raji cells exposed to EcDAF-MP with untreated controls using immunofluorescent detection of DAF with murine antihuman DAF mAb (MBC1) followed by an Alexa-Fluor-488 conjugated goat antimouse IgG. Binding of the modified protein was clearly evident following washing of the cells, whereas controls showed much lower background fluorescence. Figure 6, C and D, compares binding of EcDAF-MP and unmodified EcDAF to guinea pig erythrocytes (which, unlike Raji cells, lack human DAF). A punctate pattern of distribution suggestive of localization in lipid rafts (Simons and Ikonen 1997) was observed for EcDAF-MP but not for the unmodified protein.

Initial crystallization screening was performed using commercially available screens, and resulted in small crystals in condition 43 of Crystal Screen 1 (Molecular Dimensions Ltd. [20% mPEG4K, 0.2 M ammonium sulphate]).

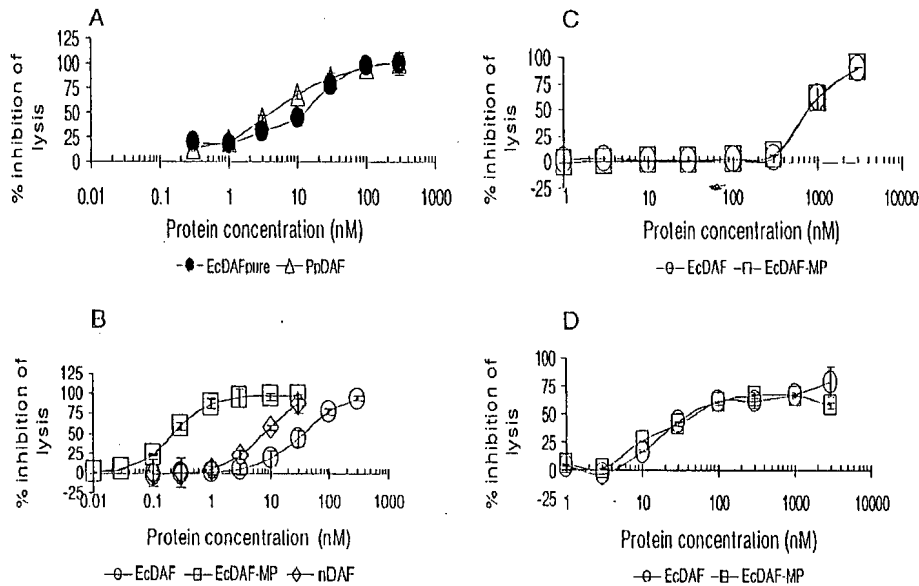


Figure 5. (A) Comparison of the activity of EcDAF (filled circles) and PpDAF (open triangles) in a classical pathway antihemolytic assay. (B) Comparison of EcDAF (open circles) with EcDAF-MP (open squares) and nDAF (open diamonds) in the classical pathway antihemolytic assay. (C) Comparison of EcDAF (open circles) with EcDAF-MP (open squares) in the alternative pathway hemolytic assay at 1 in 8 serum dilution, and (D) 1 in 16 serum dilution.

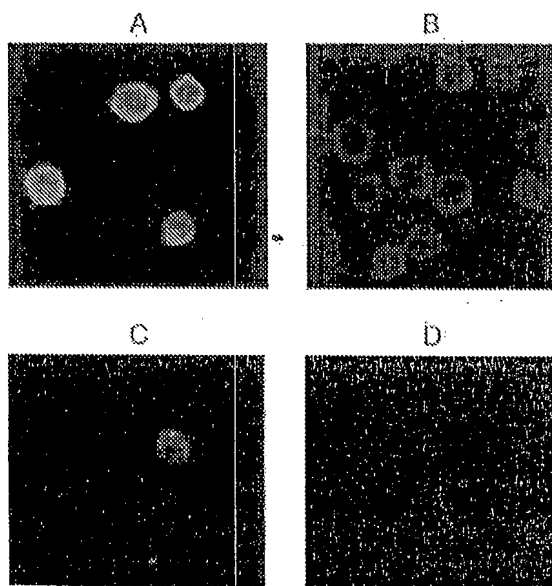


Figure 6. EcdAF binding to cells, detected with murine anti-hDAF mAb and AlexaFluor 488 labeled anti-mIgG1. Magnification $\times 40$. (A) Raji cells following exposure to $1 \mu\text{M}$ EcdAF-MP; (B) Raji cells + control (no agent); (C) guinea pig erythrocytes + EcdAF-MP; (D) guinea pig erythrocytes + EcdAF.

However, several optimization steps were required before diffraction quality crystals could be obtained. First, it was observed that minor changes to the chemical composition of the mother liquor as well as growth at low temperatures (2°C) favored crystal formation. Second, growth conditions had to be altered to allow cryoprotection (Garman 1999) of the crystals. A number of cryoprotection protocols were investigated, but the most successful approach was to incorporate glycerol cryoprotectant into the mother liquor and grow crystals under these conditions. However, high glycerol concentrations ($>10\%$) drastically reduced the number of crystals obtained. Third, the method of seeding (Stura 1991) allowed for an increase in the frequency of crystal appearance as well as limited control over the size of crystals grown (see Fig. 7). The crystals belonged to the space group P1 and diffracted to a maximum resolution of $2.2 \text{ \AA}$. Table 1 gives unit cell parameters for three typical crystals as well as data collection and processing statistics for three typical data sets.

Discussion

We have cloned and expressed a recombinant form of the four SCRs of human DAF as inclusion bodies in *E. coli* and then isolated, refolded, and purified it to produce a functional protein that inhibits complement activation in vitro at

a potency comparable with soluble DAF expressed in a eukaryotic system (*P. pastoris*). The ability to produce biologically active multi-SCR domains in bacteria was first demonstrated for a 3-SCR fragment of human CR1 (Dodd et al. 1995) and then extended to a 4-SCR unit from the same protein (Mossakowska et al. 1999) and a 4-SCR form of rat Crry (Fraser et al. 2002). The refolding protocol employed may well be general for multi-SCR proteins but the practical limit on the number of SCRs in such constructs has not yet been determined. In the case of human DAF, the recombinant material described here (EcdAF) was also able to form regular crystals that diffracted to $2.2 \text{ \AA}$, implying that EcdAF is a homogenous species and is likely to be correctly folded.

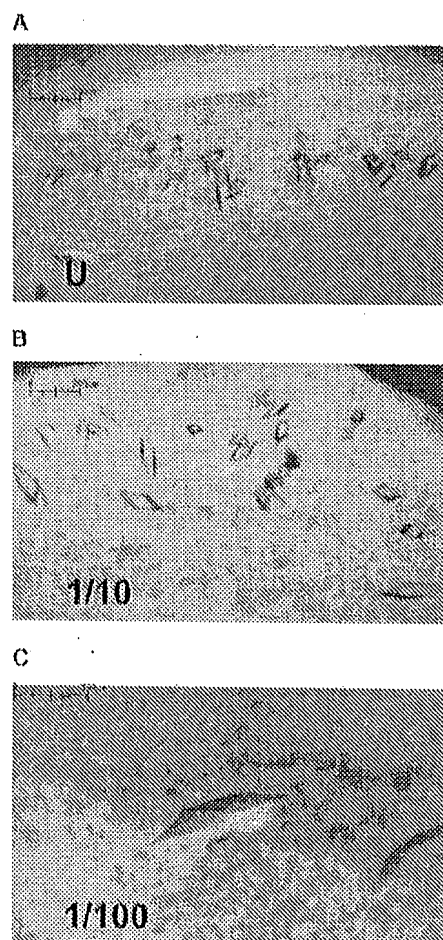


Figure 7. Effect of seeding on DAF crystal growth: (A) crystals grown from an undiluted seeding stock, (B) 10-fold diluted stock, (C) 100-fold diluted stock. The largest crystal at center of insert (C) is $210 \mu\text{m}$ in the longest dimension.

Table 1. Data collection and processing parameters for DAF crystals

	Data set 1	Data set 2	Data set 3
Collection parameters			
Detector	ADSC Q4 CCD	ADSC	MAR345
Source	ESRF beamline ID14-2	Daresbury beamline 9.6	Inhouse Rigaku RU2000 rotating
Oscillation angle (°)	1	1.5	1
Crystal to detector	220	200	260/170
Exposure time (sec)	45	90	600/1200
Wavelength (Å)	0.933	0.870	1.542
Processing parameters			
Unit cell parameters ^a	53.1, 51.9, 64.1	46.7, 55.1, 61.8, 88.8	46.7, 55.1, 61.6, 88.8
Space group	P1	P1	P1
Resolution range (outer)	39.5–2.8 (2.9–2.8)	16.0–2.2 (2.32–2.2)	17–2.3 (2.42–2.3)
Observed reflections	232,001	543,567	722,760
Unique Reflections	14,573	26,342	23,375
Completeness (outer)	99.0 (98.4)	93.5 (93.1)	94.4 (92.0)
R _{merge} ^b (outer shell)	13.5 (38.8)	9.6 (36.3)	8.3 (16.4)
I/σI (outer shell)	15.4 (3.5)	6.3 (1.9)	6.1 (4.0)

^a Dimensions a, b, c given in Å, and α, β, γ given in degrees.

^b $R_{\text{merge}}(I) = \frac{\sum_h \sum_i |I_h - I_{hi}|}{\sum_h \sum_i I_{hi}} \times 100.$

I_h is the weighted mean measured intensity of the observations *I_{hi}*, in which the intensities of the symmetry related reflections, which should be the same, are compared. *R_{merge}* (*I*) gives an estimate of their disagreement.

Our results are consistent with previous conclusions regarding the roles of both the S/T rich membrane-proximal region (Fodor et al. 1995) and the N-glycan (Brodbeck et al. 2000), neither of which appear to be essential for the complement-regulatory function of nonmembrane bound DAF. EcDAF was active in both classical and alternative pathway decay-acceleration and antihemolytic assays. In the case of the hemolytic assays, the apparent potency of the protein was lower against the alternative pathway than the classical pathway, whereas in the decay acceleration assay, the reverse was true. This can be attributed to the strong dependence of the hemolytic assays on serum concentration (see Fig. 5C,D).

We have also modified EcDAF by site-specific attachment of a membrane localizing peptide at the C-terminus and EcDAF-MP also bound to cell surfaces in vitro under conditions where EcDAF itself did not bind. Under the assay conditions used, the soluble product (EcDAF-MP) had a classical pathway antihemolytic potency apparently greater than that of native GPI-anchored DAF, although this comparison may be affected by the use of detergent to solubilize nDAF. DAF is thus added to a growing list of proteins (including complement regulatory proteins such as CR1 fragments (Smith and Smith 2001), rat Crry (Fraser et al. 2002), and CD59 (Fraser et al. 2003), to which GPI anchor-like membrane localizing properties can be conferred by an efficient posttranslational modification process using synthetic peptides. We observed that although the attachment of the membrane-localizing peptide produced a large (>100-fold) increase in potency against the classical pathway (Fig.

5), no such effect was seen against the alternative pathway. To establish if this was due to the higher concentrations of serum used in the alternative pathway assays preventing membrane binding, “wash” experiments were performed in which rabbit or guinea pig erythrocytes were loaded with EcDAF-MP in the absence of serum, washed, and then challenged. The results suggested that under the conditions of the alternative pathway assay (unlike those of the classical pathway assay), EcDAF-MP was not able to bind sufficiently strongly to the target erythrocytes to confer intrinsic protection against challenge by a relatively high concentration of serum. Other studies have suggested that the effect of serum is largely mediated by albumin and can be circumvented by altering the targeting tail structure (D. Esser, L. Fiedler, J.R. Betley, I. Dodd, P.J.E. Rowling, G.P. Smith, R.G. Oldroyd, L. Affleck, S. Ridley, and R.A.G. Smith, unpubl.). The effect of albumin does not appear to abrogate the activity of membrane-targeted complement regulators delivered to sites of disease in vivo (Linton et al. 2000; Pratt et al. 2003).

DAF has significant potential as a therapeutic agent (Moran et al. 1992), and recent studies have demonstrated therapeutic utility for DAF fusions with immunoglobulin Fc domains (Harris et al. 2002; Yanagawa et al. 2003). However, except in transgenic systems (Focke et al. 2002), the potential for an intrinsically acting membrane bound form of DAF has not been realized. In part, this is because of the difficulties in obtaining sufficient quantities of nDAF for evaluation, and in part because of the relatively low potency of non-GPI-anchored forms (Moran et al. 1992; Walter et al.

1992). The strategy adopted here circumvents these problems first by providing for a potentially large-scale and low-cost production system, and second, because of the increased potency of the modified soluble derivatives. In addition, myristoylated peptide modifications permit transfer of modified materials from the plasma phase to vascular sites in vivo (Smith 2002) and the distribution of such modified proteins in vivo can be manipulated by varying the structure of the peptide "tail" (D. Esser, L. Fiedler, J.R. Belley, I. Dodd, P.J.E. Rowling, G.P. Smith, R.G. Oldroyd, L. Affleck, S. Ridley, and R.A.G. Smith, unpubl.).

The crystallization of a biologically active four-domain form of DAF suggests that this material is correctly folded, and the collection of high-quality and reproducible diffraction data (Table 1) has permitted the determination of an atomic structure for this protein. This has been extended to the modeling of the complete extracellular region of the molecule as well as its interaction with Factor B (Lukacik et al. 2004). The complete structural description also sheds light on the function of the MP targeting peptides. Despite being much smaller than the combined GPI anchor and S/T region, the MP peptide appears to be capable of localizing the SCRs of DAF in a functionally efficient orientation on the outer cell membrane. Figure 8 compares full-length DAF (based on the atomic structure including the S/T-rich region) with EcDAF-MP, in which that region is replaced by the MP peptide. Clearly, the region of DAF implicated in regulation of the classical pathway convertases (SCRs 2 and 3; Brodbeck et al. 1996) is spaced very differently from the membrane surface in the two cases. Although the S/T rich region may be assumed to be flexible, the small size of the MP peptide (at least half of which is probably in direct contact with the membrane), provides a constraint on the distance of the C2a component of the classical pathway C3 convertase from cell surfaces.

In conclusion, the atomic structure of DAF, when combined with the use of membrane-orienting probes, significantly extends our knowledge of structure-function relationships that have previously been based solely on partial structures of soluble fragments (e.g., Uhrinova et al. 2003; Williams et al. 2003). Derivatives of the EcDAF-MP type also provide powerful tools for probing the biological roles and therapeutic potential of DAF.

Materials and methods

Proteins and chemicals

GPI-anchored DAF (nDAF) was prepared as previously described (Morgan 2000). PpDAF was prepared by the method of Powell et al. (1997). SCRs(1–3) of CR1 were prepared as described by Mossakowska et al. (1999).

Chemicals and reagents were supplied by Sigma-Aldrich unless otherwise stated.

Phosphate-buffered saline (Dulbecco A) was from Oxoid Ltd. Sodium chloride and Tween 20 were from Merck; EDTA and urea

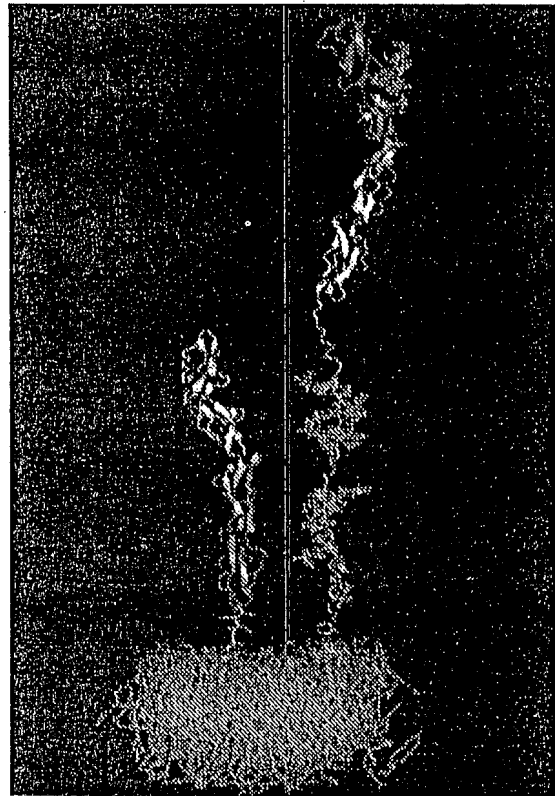


Figure 8. A comparison of full-length DAF (right) with EcDAF-MP (left) based on the atomic structure including the modeled S/T-rich region (Lukacik et al. 2001). In EcDAF-MP, the S/T region is replaced by the MP peptide. The structures are modeled upon a simulated phospholipid bilayer. Both S/T and MP peptides are depicted as maximally extended (the latter is proposed to be in contact with the bilayer through the myristoyl group and at least the first three lysine residues).

were from Prolabo; Novex 4%–12% Bis Tris Nupage gels, and Mark 12 protein molecular weight standards were from Invitrogen.

N-myristoyl GlySerSerLysSerProSerLysLysLysLysLysLysPro-GlyAspCys-(S-2-thiopyridyl) carboxamide (APT542) was prepared as previously described (Smith et al. 1998; Smith and Smith 2001). Tris-(2-carboxyethyl) phosphine (TCEP) was supplied by Pierce.

Alexa488 conjugated goat antimouse IgG was from Molecular Probes.

Cells and serum

Rabbit antibody sensitized sheep erythrocytes were purchased from Diamidex. Rabbit and guinea pig erythrocytes in Alsever's buffer were purchased from TCS Biologicals. Normal human serum was prepared from a pool of volunteers by standard methods.

Cloning and expression

The gene encoding the entire human DAF sequence was obtained by RT-PCR from a human brain total RNA preparation (Origene

Inc.) using primers: (GCATATGACCGTCGCGCGCCGAGC) DAF-F and (GGAATTCCTAAGTCAGCAAGCCCATGGTTACT) DAF-R.

The product of the PCR was T-cloned into the plasmid pUC57/1 (MBI Fermentas) and authenticated by nucleotide sequence analysis.

A fragment of the full-length gene encompassing the four SCRs of DAF, along with an N-terminal Met-Gln addition and a C-terminal Cys (MQ)₃₅-G₂₈₅C was generated from this plasmid template by PCR using primers DAF1-IF (GCATATGACGAGAC TGTGGCCTGCCGCGCGGACGTT) and DAF4R (GGAATTC TATCAGCATCCTCTGCATTCAGGTGGTGG) that incorporated NdeI and EcoRI sites for subsequent subcloning steps, and the product T-cloned. The DAF1-4 DNA fragment was inserted, and excised from this plasmid using the NdeI and EcoRI restriction sites incorporated into the primers. This was followed by ligation into the pET-26b(+) vector downstream of the T7 RNA polymerase promoter (Novagen) for expression in *E. coli* (see below).

Expression was carried out in *E. coli* UT5600(DE3). A 25-mL culture in LB-kanamycin was grown overnight at 37°C. Ten milliliters of the overnight culture was then transferred into 1 liter of EZMix (Sigma)-kanamycin in a 5L-culture flask. The culture was grown for 75 min (OD₆₀₀ = 0.6) and then induced by adding 1 mL of 1 M IPTG. The induced culture was grown for a further 3 h, after which it was centrifuged (9000g for 30 min) to obtain bacterial pellets.

Isolation of inclusion bodies, refolding and purification

Frozen cell pellets were resuspended in 20 mM Tris-HCl, 1 mM EDTA (pH 8.0 [40 mL]), and passed through a homogenizer twice (Emulsiflex-C5, Avestin, 12 kpsi). The homogenate was centrifuged (10,000g/4°C/30 min) and the supernatant discarded. The pellet containing the inclusion bodies was washed twice in PBS/0.05% (w/v) Tween 20, centrifuged (10,000g/4°C/30 min) and the supernatants discarded. The pellet was resuspended in 8 M urea, 25 mM dithiothreitol, 1 mM EDTA, 0.1 M Tris (pH 8.0 [10 mL]) to facilitate solubilization. After 2 h (room temperature/gentle stirring) the pH of the solution was adjusted to 3.0–4.0 with HCl, and the solution was centrifuged (10,000g/4°C/30 min) before dialysis in 6 M urea, 10 mM HCl (pH 3.0–4.0). After dialysis, the major contaminant (OmpA) precipitated and was removed by centrifugation (10,000g/4°C/30 min).

Folding of solubilized protein was carried out in 0.02 M ethanolamine, 1 mM EDTA, 0.5 M arginine, 1 mM cysteine, 2 mM cystine (pH 11.0) using rapid dilution (1:50 v/v) with three additions of the same volume over a 32-h period. Additions were drop-wise with stirring, and then the solution, which remained clear, was left static at 4°C. This material was ultrafiltered using an Amicon YM10 membrane (Millipore), the retentate (20 mL) was centrifuged (20,000g/4°C/20 min). Soluble EcDAF was buffer-exchanged into PBS using a G25 Sephadex column (G25 Sephadex medium grade, Amersham Biosciences) by monitoring the A280 of the eluate and collecting the V₀ fraction. The resulting EcDAF preparation was approximately 80% pure, as determined by SDS-PAGE using staining with Coomassie brilliant blue R250 (Fig. 2, lane 8).

For crystallization studies, the EcDAF preparation was buffer exchanged into 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and further purified by gel filtration on Hi Load 26/60 Superdex S75 (Amersham Biosciences) run at 18°C.

The resultant preparation was >95% pure as determined by SDS-PAGE/CBBR250.

Protein concentrations

Protein concentrations were determined by quantitative SDS-PAGE/CBBR250 analysis (UVP Gel Documentation System GDS8000) and analyzed using GelWorks 1D Analysis software using an in-house protein standard and/or by A280 readings using a calculated molar extinction coefficient of 38,880 M⁻¹cm⁻¹ for EcDAF (Protein) or 36,840 M⁻¹cm⁻¹ (ProtParam; Gill and von Hippel 1989). Concentration of nDAF was determined by dye binding assay using BSA as a standard (Bradford 1976).

Modification with APT542

EcDAF at 2 mg/mL (71 μM) in PBS Dulbeccos A was treated with threefold molar excess of TCEP and incubated overnight at room temperature to cleave the *exo*-disulfide bonded between C-terminal protein cysteine and cysteine derived from the refold buffer. DAF was then incubated with fivefold molar excess of the myristoylated peptide APT542 for 2 h at room temperature. In certain experiments, residual APT542 was removed by gel filtration using PD-10 G25 Sephadex columns as recommended by the manufacturer (Amersham Biosciences).

Assays

For studies of classical pathway activation, sensitized sheep erythrocytes (100 μL) were incubated with DAF proteins (50 μL) and 1 in 100 diluted normal human serum (50 μL) added for 1 h at 37°C. Cells were pelleted at 430 ref for 3 min and lysis was determined by measuring the OD of the supernatant at 410 nm. The diluent for this assay was: 0.1 M HEPES, 0.15 M NaCl, 0.1% gelatin (pH 7.4), CHAPS (3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate) (0.05% w/v) was used in the diluent for assay of nDAF to retain solubility. For studies of alternative pathway activation, rabbit erythrocytes in Alsever's buffer were washed and diluted 1 in 50 (~5 × 10⁶ cells/mL), 50 μL of cells were incubated with DAF proteins (50 μL), and 1 in 8 normal human serum (100 μL) for 1 h at 37°C. Cells were pelleted at 430 ref for 3 min and lysis was determined by measuring optical density at 410 nm of the supernatant. The alternative pathway diluent was 0.3 M HEPES, 0.15 M NaCl, 8 mM EGTA, 5 mM MgCl₂, 0.1% gelatin (pH 7.4). The "wash" variant of this assay (Fraser et al. 2002) was carried out by preincubation of the cells with varying concentrations of an agent in the absence of serum, pelleting, and resuspension three times in the above buffer, followed by serum challenge.

Antihemolytic activity in both assays was expressed as the concentration of the agent giving 50% of the total cell lysis (IC₅₀).

Fluid-phase assays of decay acceleration were performed as described by Nickells and Atkinson (1997) using purified components (Advanced Research Technologies) and C3a immunoassay (Quidel).

Crystallization and diffraction data collection

A number of crystallization conditions were initially screened by the sitting drop vapor diffusion method in Linbro plates.

After several rounds of optimization the following protocol was devised to reliably obtain diffraction quality crystals: 0.5 μL of well solution and 0.5 μL (9.6 mg/mL) of protein were mixed on a polypropylene microbridge (Harlos 1992). The well solution contained 0.2 M ammonium sulfate, 20% w/v methoxy polyethylene glycol 5000, 0.1 M sodium acetate (pH 4.6), 10% glycerol. The

1 μ L drop was allowed to pre-equilibrate at 2°C in a sealed well of a Linbro plate for 12 h before seeding (Stura 1991). Seeding was performed by transferring dilutions of a crushed crystal stock into pre-equilibrated drops. Approximately 15 DAF crystals in a 1- μ L drop were crushed, and the drop contents were diluted 10-fold and 100-fold. An animal hair fiber was used to transfer the diluted stocks into a pre-equilibrated drop. This method increased the frequency of crystal appearance and allowed for growth of larger crystals. The largest crystals could be obtained by using more dilute stocks (e.g., 100-fold). Diffraction quality crystals were obtained by this method in approximately 6 days.

Immediately prior to the diffraction experiment, crystals grown in the presence of 10% glycerol were transferred to a solution replicating mother liquor conditions but with a higher (16%) glycerol concentration. This glycerol concentration was just sufficient to achieve full cryoprotection of the crystal. The crystals were then flash frozen in a cold stream of nitrogen gas at 100 K. X-ray diffraction data were collected on an ADSC Q4 CCD-based detector at beamline ID14-2 European Synchrotron Radiation Facility (ESRF), Grenoble, for data set 1; on an ADSC detector at beamline 9.6, Synchrotron Radiation Source (SRS), Daresbury, UK for data set 2, and on a MAR345 detector utilizing a Rigaku RU200H rotating anode X-ray generator for data set 3 (University of Oxford).

A number of data sets were collected from these crystals. Data sets were integrated and scaled using either the HKL package (Otwinowski and Minor 1997) or programs MOSFLM and SCALA available as part of the CCP4 suite of programs (Collaborative Computational Project 1994).

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