Active site titration of bovine β-trypsin by N^{α}-(N,N-dimethylcarbamoyl)-α-aza-lysine p-nitrophenyl ester: kinetic and crystallographic analysis

Patrizia Sartori[a], Kristina Djinovic Carugo[a], Raffaella Ferraccioli[b], Gianni Balliano[c], Paola Milla[c], Paolo Ascenzi[d], Martino Bolognesi[a,e,*]

[a]Dipartimento di Genetica e Microbiologia, Universita' di Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy
[b]CNR, Centro Sintesi e Stereochimica di Speciali Sistemi Organici, Via Golgi 19, 20133 Milano, Italy
[c]Istituto di Chimica Farmaceutica Applicata, Universita'di Torino, Corso Raffaello 31, 10125 Torino, Italy
[d]Dipartimento di Scienza e Tecnologia del Farmaco, Universita' di Torino, Via Pietro Giuria 9, 10125 Torino, Italy
[e]Centro Biotecnologie Avanzate and Dipartimento di Fisica, Universita' di Genova, Viale Benedetto XV 10, 16132 Genova, Italy

Received 6 October 1994; revised version received 28 November 1994

Abstract **Kinetics of bovine β-trypsin (trypsin) with the N^{α}-(N,N-dimethylcarbamoyl)-α-aza-lysine p-nitrophenyl ester (Dmc-azaLys-ONp) was obtained at pH 6.2 and 21.0°C. Dmc-azaLys-ONp shows the characteristics of an optimal active site titrant in that it (i) gives titrations in a short time, (ii) is a stable and soluble compound with a stoichiometric reaction that is easily and directly detectable, and (iii) allows titrations over a wide range of enzyme concentration. Moreover, the three-dimensional structure of the trypsin · N^{α}-(N,N-dimet hylcarbamoyl)-α-aza-lysine acyl · enzyme adduct has been solved by X-ray crystallography at 2.0 Å resolution (R = 0.145). The Dmc-azaLys moiety of the active site titrant is sited in the serine proteinase reaction center, and is covalently linked to the OG atom of the Ser^{195} catalytic residue.**

Key words: Bovine β-trypsin; N^{α}-(N,N-Dimethylcarbamoyl)-α-aza-lysine p-nitrophenyl ester; Dmc-azaLys-ONp; Enzyme kinetics; Active enzyme concentration (determination of); Acyl·enzyme adduct (X-ray crystal structure of)

1. Introduction

Serine proteinase concentration may easily be determined by titration with reactants (i.e. p-nitrophenyl esters) which bind stoichiometrically to the enzyme active site, forming a stable acyl·enzyme adduct, with the simultaneous release of the chromophoric group (i.e. p-nitrophenol). The most commonly used active site titrant for serine proteinases belonging to the chymotrypsin superfamily and acting on cationic substrates (i.e. bovine β-trypsin (trypsin)) is p-nitrophenyl p-guanidinobenzoate (pN-pGB) which, however, does not fit the specificity requirements as efficiently as p-nitrophenyl esters of amino acids do. On the other hand, N-α-carbobenzoxy-L-arginine p-nitrophenyl ester (Z-Arg-ONp) and N-α-carbobenzoxy-L-lysine p-nitrophenyl ester (Z-Lys-ONp) form unstable acyl·enzyme adducts with serine proteinases [1].

Previous results indicated that p-nitrophenyl esters of aza-amino acids are specific chromogenic reactants for serine proteinases belonging to the chymotrypsin and subtilisin superfamilies [2,3]. Thus, the potentially wide use of N^{α}-(N,N-dimethylcarbamoyl)-α-aza-lysine p-nitrophenyl ester (Dmc-azaLys-ONp; see Fig. 1) as a specific chromogenic serine proteinase active site titrant stimulated our interest in adapting and improving methods [1] for the determination of the enzyme concentration.

The present study reports (i) kinetics of trypsin with Dmc-azaLys-ONp, which shows the characteristics of an optimal chromogenic serine proteinase active site titrant; and (ii) the X-ray crystal structure of the trypsin·Dmc-azaLys acyl·enzyme adduct, determined at 2.0 Å resolution and refined to a crystallographic R-factor value of 0.145.

2. Materials and methods

Trypsin, treated with diphenylcarbamyl chloride in order to abolish chymotryptic activity, was purified from commercial enzyme preparations (from Sigma Chemical Co., St. Louis, MO, USA) as previously detailed [4]. Dmc-azaLys-ONp was synthesized as previously reported [3].

The kinetics for the trypsin catalyzed hydrolysis of Dmc-azaLys-ONp was obtained under conditions where the Dmc-azaLys-ONp concentration ($[S_0]$) exceeds the trypsin concentration ($[E_0]$) by at least five-fold (i.e. $[S_0] \geq 5 \times [E_0]$) [1]. The optical density increase on hydrolysis of Dmc-azaLys-ONp (i.e. on release of p-nitrophenol) was followed spectrophotometrically at 360 nm ($\delta\varepsilon$ = 5.2 $mM^{-1} \cdot cm^{-1}$ [5]), pH 6.2 (0.1 M phosphate buffer) and 21.0°C, with a Jasco J-500 apparatus equipped with the SFC-5 rapid-mixing stopped-flow accessory [1].

Kinetic parameters for the trypsin catalyzed hydrolysis of Dmc-azaLys-ONp were obtained from the experimental data according to the standard treatment of the minimum three-step catalytic mechanism of serine proteinases (Scheme I) [1]:

$$E + S \overset{K_s}{\rightleftharpoons} E \cdot S \overset{k_{+2}}{\longrightarrow} \underset{\underset{P_1}{+}}{E \cdot P} \overset{k_{+3}}{\longrightarrow} E + P_2 \qquad \text{(I)}$$

where E is trypsin, S is Dmc-azaLys-ONp, E · S is the reversible rapidly formed enzyme·Dmc-azaLys-ONp complex, E · P is the trypsin·Dmc-azaLys acyl·enzyme adduct, P_1 and P_2 are p-nitrophenol and Dmc-azaLys, respectively, K_s is the fast pre-equilibrium constant, k_{+2} is the acylation rate constant, and k_{+3} is the deacylation rate constant.

Crystals of the trypsin·Dmc-azaLys acyl·enzyme adduct were obtained by soaking of the free enzyme crystals [6] in 2.5 M ammonium sulfate solution, pH 8.0, containing 25 mM Dmc-azaLys-ONp, for 1 day at 4.0°C. Diffraction data to 2.0 Å resolution were collected on a Rigaku R-axis-II imaging plate system (Molecular Structure Corp., Houston, TX, USA). A total of 55,989 intensities were collected and reduced to 18,421 independent structure factors (94.6% completeness

*Corresponding author. Fax: (39) (382) 528 496.

Abbreviations: trypsin, bovine β-trypsin; pN-pGB, p-nitrophenyl p-guanidinobenzoate; Z-Arg-ONp, N-α-carbobenzoxy-L-arginine p-nitrophenyl ester; Z-Lys-ONp, N-α-carbobenzoxy-L-lysine p-nitrophenyl ester; Dmc-azaLys-ONp, N^{α}-(N,N-dimethylcarbamoyl)-α-aza-lysine p-nitrophenyl ester; Dmc-azaLys, N^{α}-(N,N-dimethylcarbamoyl)-α-azalysine.

SSDI 0014-5793(94)01366-7

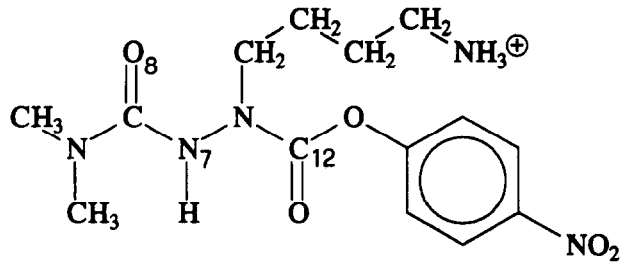

Fig. 1. Chemical structure of Dmc-azaLys-ONp. Only the N(7), O(8) and C(12) atoms of the active site titrant, relevant for the discussion, have been labeled.

in the 15.0– 2.0 Å resolution range; *R*-merge = 2.9%), using Molecular Structure Corp. propietary software, based on the MOSFLM program package [7]. The structure of the trypsin·Dmc-azaLys acyl·enzyme adduct was analyzed by difference Fourier techniques, using the Brookhaven Protein Data Bank coordinates file 1TDL [8] for trypsin atomic coordinates and starting phase calculation (*R*-factor = 0.294). The crystallographic refinement of the structure was performed using the program TNT [9] and alternating map inspection/model correction cycles, using FRODO [10]. After convergence in the refinement was reached (as estimated from drop in the *R*-factor and shifts of the refined parameters), a difference Fourier map, calculated with (F_o-F_c) coefficients, did not show significant structural features above the 3.0 σ (electron density) noise level. The final model consists of 1,629 trypsin atoms, 14 non-hydrogenic atoms from the bound Dmc-azaLys,and 152 solvent molecules; the corresponding *R*-factor, in the 15.0 to 2.0 Å resolution range, has a value of 0.145, with nearly ideal values for the model stereochemical parameters.

3. Results and discussion

The trypsin catalyzed hydrolysis of Dmc-azaLys-ONp shows an instantaneous release of *p*-nitrophenol (i.e. P_1; see Eq. I), which is not followed by the steady-state reaction commonly observed for substrates; in fact, the trypsin·Dmc-azaLys acyl·enzyme adduct decays less than 5% over one day (i.e. $k_{+3} < 6 \times 10^{-7}$ s^{-1}; see Scheme I). Such a process may be described by the classical equation (arising from Scheme I) [1]:

$$[P_1] = \pi \cdot (1-e^{-kt}) \quad (1)$$

where $[P_1]$ is the *p*-nitrophenol (i.e. titrant) concentration,

$$\pi = [E_0] \quad (2)$$

is the total amplitude of the time–course for the instantaneous release of *p*-nitrophenol, which corresponds to the molar concentration of *p*-nitrophenol released (i.e. to the molar concentration of the active enzyme) (see Scheme I and Fig. 2A), and

$$k = k_{+2}/\{1+K_s/[S_0]\} \quad (3)$$

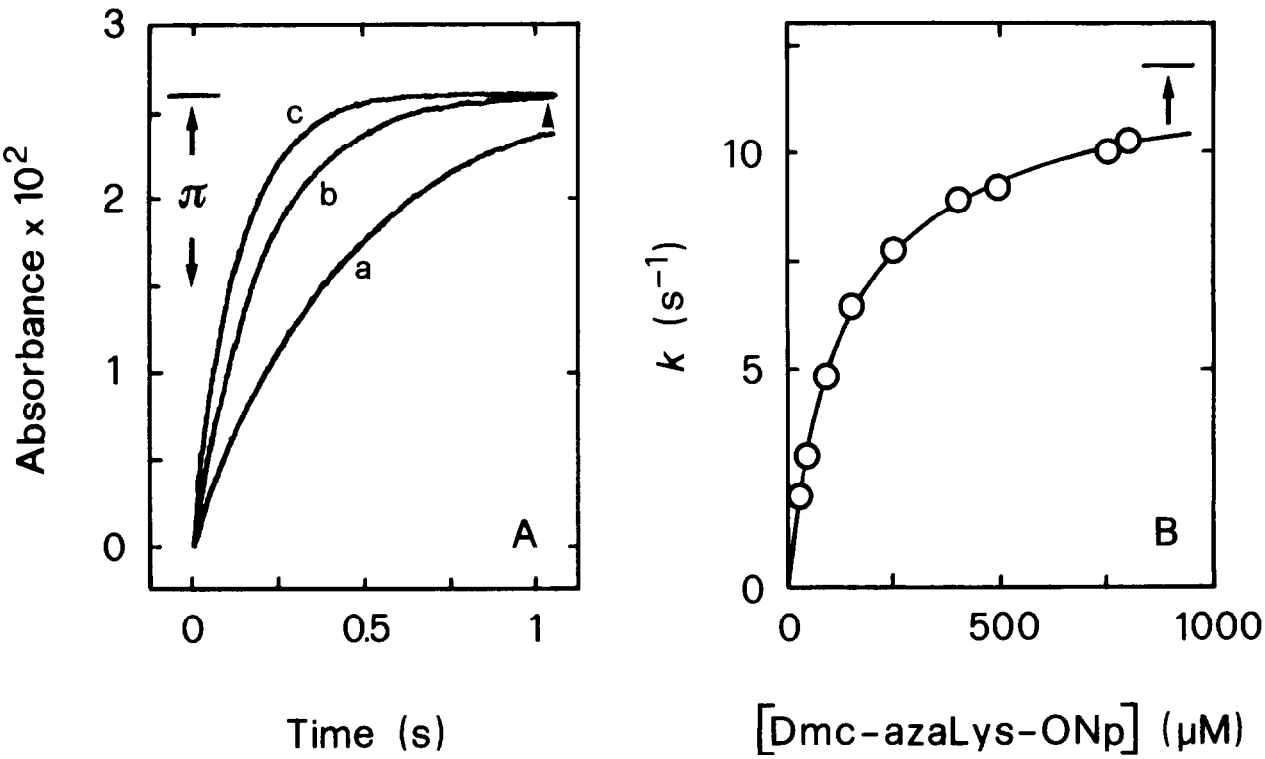

Fig. 2. (A) Time–course of *p*-nitrophenol release during the trypsin catalyzed hydrolysis of Dmc-azaLys-ONp at pH 6.2 and 21.0°C, with $[S_0] \geq 5 \times [E_0]$. Kinetics of *p*-nitrophenol release were obtained at $[S_0] = 3.0 \times 10^1$ μM (a), 1.0×10^2 μM (b) and 2.5×10^2 μM (c.); $[E_0]$ was 5.0 μM. The total amplitude of the time–course of *p*-nitrophenol release (π; see Eq. 2) corresponds to an optical absorbance change of 2.6×10^{-2} (i.e. to an active trypsin concentration of 5.0 μM). Values of the apparent first order rate constant of the time–course of *p*-nitrophenol release (k; see Eq. 3) are 2.2 s^{-1} (a), 4.9 s^{-1} (b) and 7.7 s^{-1} (c). (B) Effect of the Dmc-azaLys-ONp concentration on the apparent first order rate constant (k) for the trypsin catalyzed hydrolysis of the active site titrant at pH 6.2 and 21.0°C, with $[S_0] \geq 5 \times [E_0]$. The continuous line, representing the best theoretical curve fitting the data, was calculated according to Eq. 3, with $K_s = 1.4 \times 10^2$ μM and $k_{+2} = 1.2 \times 10^1$ s^{-1}. An average S.E.M. of ± 7% was evaluated as the standard deviation for the values of K_s and k_{+2}. The arrow indicates the asymptotic value of k which corresponds to k_{+2} when $[S_0] >> K_s$ (see Eq. 3). For further details, see text.

Fig. 3. Stereo view of the trypsin·Dmc-azaLys acyl·enzyme adduct. The active site titrant is in the central part of the picture, covalently linked to the OG atom of Ser_{195}. Residues Asp_{102}, His_{57} and Ser_{195}, forming the catalytic triad, and Asp_{189}, present at the bottom of the S_1 subsite, have been labeled.

is the apparent first order rate constant of the time–course of *p*-nitrophenol release (see Fig. 2A).

Under all the experimental conditions, the time–course of *p*-nitrophenol release for the trypsin catalyzed hydrolysis of Dmc-azaLys-ONp corresponds to a single exponential decay for more than 95% of its course (see Fig. 2A).

Since trypsin contains a single active site per molecule [11], the unknown active enzyme concentration (i.e. $[E_0]$) may be directly determined from the value of π, according to Eq. 2. From the data shown in Fig. 2A, an active trypsin concentration of 5.0 μM was determined. The enzyme concentration obtained by using Dmc-azaLys-ONp is in excellent agreement with that determined (i) by titrating the same enzyme sample with *p*N-*p*GB, Z-Arg-ONp and Z-Lys-ONp [1], and (ii) spectrophotometrically from the optical absorption at 280 nm [4]. The total amplitude of the spectrophotometric signal at 360 nm (π) allows the determination of the active enzyme concentration between 1 μM and 300 μM, using Dmc-azaLys-ONp. Over this trypsin concentration range, π is linearly dependent on $[E_0]$, as expected from Eq. 2.

Fig. 2B shows the effect of the active site titrant concentration on the apparent first order rate constant (k) for the trypsin catalyzed hydrolysis of Dmc-azaLys-ONp. The continuous line, representing the best theoretical curve fitting the data, was calculated according to Eq. 3, with $K_s = 1.4 \times 10^2$ μM and $k_{+2} = 1.2 \times 10^1$ s^{-1}.

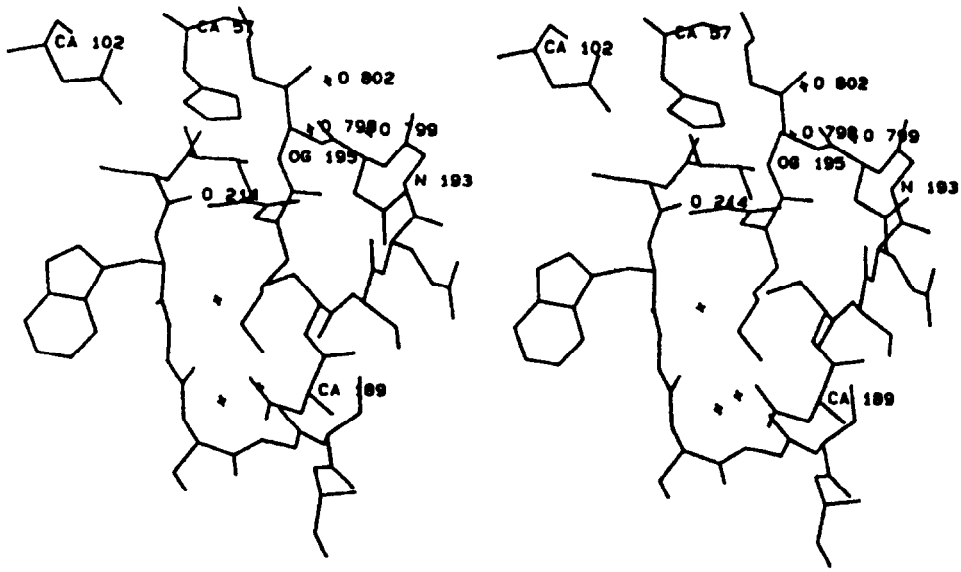

Fig. 4. Stereo view of the acyl·trypsin active site in the presence of the bound Dmc-azaLys, which is evident in the center of the picture, covalently linked to the OG atom of Ser_{195}. Residues Asp_{102}, His_{57} and Ser_{195}, forming the catalytic triad, Asp_{189}, present at the bottom of the S_1 subsite, Gly_{193} and Ser_{214}, as well as W798, W799 and W802 water molecules, have been labeled.

The high stability of the trypsin·Dmc-azaLys acyl·enzyme adduct, expressed by the very low value of k_{+3} ($< 6 \times 10^{-7}$ s^{-1}), may be related to the following crystallographic observations. The Dmc-azaLys substructure is completely and unambigously defined by its observed electron density. As shown in Figs. 3 and 4, the active site titrant is sited in the trypsin active center, and is covalently bound to the OG atom of the Ser_{195} catalytic residue. Despite the presence of a trigonal planar nitrogen atom at the inhibitor aza-center, the lysine side chain of the active site titrant is accommodated in the trypsin primary specificity cleft (S_1), forming a hydrogen bond and an ion pair with the Asp_{189} residue, and trapping a water molecule (W794) at the dead end of the S_1 subsite [12]. The acyl bond to the Ser_{195} OG atom is oriented in such a way that the carbonyl O atom is pointing towards the peptide Gly_{193} N atom (3.23 Å). On the otherhand, the *N,N*-dimethylcarbamoyl moiety of Dmc-azaLys is oriented towards the central part of the proteinase active site, being in contact with the imidazole ring of His_{57}. A hydrogen bond connects the N(7) atom of Dmc-azaLys (see Fig. 1) with the carbonyl O atom of Ser_{214} (2.66 Å). Moreover, the His_{57} NE2 atom is hydrogen bonded to the OG atom of the acylated Ser_{195} residue (2.95 Å). Two highly ordered water molecules are located in the neighborhood of the scissile acyl bond. The W798 water molecule is at hydrogen bonding distance from the His_{57} NE2 atom (2.77 Å), from the Dmc-azaLys O(8) atom (2.75 Å; see Fig. 4), and from the nearby W799 and W802 water molecules (2.74 Å and 3.02 Å, respectively). An additional hydrogen bonded interaction, to the Ser_{195} OG atom (3.04 Å), can contribute to the location of the W798 water molecule. Moreover, the W799 water molecule is at 2.89 Å from the peptidyl Gly_{193} N atom, and at 3.15 Å from the carbonyl O atom of Phe_{41}.

As shown in Fig. 4, the trypsin active site/Dmc-azaLys/solvent (particularly, W798, W799 and W802) system appears to be trapped in a low reactivity configuration, the scissile acyl bond being exposed to solvent only on one side (upper in Fig. 4). The W798 water molecule, present in the acylated enzyme, may be seen as a potential nucleophile for the deacylation reaction. However, the location of the W798 water molecule, withdrawn from the scissile bond carbonyl C(12) atom, is firmly defined by a strong hydrogen bonded network, which includes the enzyme and the active site titrant atoms. Onset of the deacylation process would require this structural organization to relax substantially, in order to shorten the nucleophile-to-electrophile distance of about 1.5 Å, the observed distance between W798 and the Dmc-azaLys C(12) atom (see Figs. 1 and 4) being 3.71 Å.

X-ray crystal studies concerning slow deacylating compounds covalently bound to the Ser_{195} OG atom of trypsin, bovine chymotrypsin and human leukocyte elastase have been recently reported [6,13,14]. In spite of the different chemical nature of the reactant, the structural organization of the acyl·enzyme adduct is closely reminiscent of that observed for the stable trypsin·Dmc-azaLys complex.

As a whole, the enzyme assay here described offers a reliable, sensitive and precise method for the quantitative determination of the active trypsin concentration. In fact, Dmc-azaLys-ONp shows the characteristics of an optimal specific active site titrant, in that it (i) gives titrations in a short time, (ii) is a stable and soluble compound, the stoichiometric reaction of which is easily and directly detectable, and (iii) allows titrations over a wide range of enzyme concentrations.

Acknowledgements: Authors thank Prof. G. Amiconi, Prof. C. Gallina and Prof. R. Scandurra for helpful discussions. This study was partially supported by grants from the Ministry of University, Scientific Research and Technology of Italy (MURST), from the National Research Council of Italy (CNR), and from the Centro di Studio Macromolecole Informazionali (University of Pavia, Italy).

References

[1] Ascenzi, P., Menegatti, E., Guarneri, M. and Amiconi, G. (1987) Biochim. Biophys. Acta 915, 421–425.
[2] Powers, J.C. and Harper, J.W. (1986) in: Proteinase Inhibitors (Barrett, A.J. and Salvesen, G., eds.) pp. 55–152, Elsevier, Amsterdam.
[3] Ferraccioli, R., Della Croce, P., Gallina, C., Consalvi, V. and Scandurra, R. (1991) Il Farmaco Ed. Sc. 46, 1517–1529.
[4] Luthy, J.A., Praissman, M., Finkenstadt, W.R. and Laskowski Jr., M. (1973) J. Biol. Chem. 248, 1760–1771.
[5] Ascenzi, P., Bertollini, A., Verzili, D., Brunori, M. and Antonini, E. (1980) Anal. Biochem. 103, 235–239.
[6] Djinovic Carugo, K., Rizzi, M., Fasano, M., Luisetti, M., La Rosa, C., Ascenzi, P. and Bolognesi, M. (1993) Biochem. Biophys. Res. Commun. 193, 32–39.
[7] Leslie, A.G.W., Brick, P. and Wonacott, A.J. (1986) CCP4 News 18, 33–39.
[8] Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer Jr., E.F., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–543.
[9] Tronrud, D.E., Ten Eyck, L.F. and Matthews, B.W. (1987) Acta Crystallogr. sect A, 43, 489–501.
[10] Jones, T.A. (1978) J. Appl. Crystallogr. 11, 268–272.
[11] Bode, W. and Schwager, P. (1975) J. Mol. Biol. 98, 693–717.
[12] Bode, W. and Huber, R. (1992) Eur. J. Biochem. 204, 433–451.
[13] Mangel, W.F., Singer, P.T., Cyr, D.M., Umland, T.C., Toledo, D.L., Stroud, R.M., Pflugrath, J.W. and Sweet, R.M. (1990) Biochemistry 29, 8351–8357.
[14] Singer, P.T., Smalås, A., Carty, R.P., Mangel, W.F. and Sweet, R.M. (1993) Science 259, 669–673.