Formation and characterization of a transition state complex of *Azotobacter vinelandii* nitrogenase

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Abstract A stable complex is formed between the nitrogenase proteins of *Azotobacter vinelandii*, aluminium fluoride and MgADP. All nitrogenase activities are inhibited. The complex formation was found to be reversible. An incubation at 50°C recovers nitrogenase activity. The complex has been characterized with respect to protein and nucleotide composition and redox state of the metal–sulphur clusters. Based on the inhibition by aluminium fluoride together with MgADP, it is proposed that a stable transition state complex of nitrogenase is isolated.

Key words: Nitrogenase (*Azotobacter vinelandii*); ATP-analogue; Aluminium fluoride; Molecular switch protein

1. Introduction

In the presence of MgATP and a strong reductant, nitrogenase (EC 1.18.6.1) catalyses the reduction of dinitrogen to ammonia [1,2]. The enzyme consists of two proteins: the MoFe protein (Av1), an $\alpha_2\beta_2$ tetramer of 230 kDa, and the Fe protein (Av2), a γ_2 dimer of 63 kDa. Each (independently functioning) $\alpha\beta$-half of the MoFe protein contains an Fe protein binding site and two metal–sulphur clusters: the FeMo cofactor (FeMoco) and the P-cluster [3,4]. The Fe protein contains a single [4Fe–4S] cluster and two binding sites for MgATP or MgADP [5].

The generally accepted model for the reduction of dinitrogen by nitrogenase, proposed by Thorneley and Lowe, consists of two electron transfer cycles [6]. In the Fe protein cycle the reduced Fe protein (with MgATP bound) and the MoFe protein associate which yields the active nitrogenase complex. In the nitrogenase complex a single electron is transferred from the Fe protein to the MoFe protein and MgATP is hydrolysed [7]. Hereafter the nitrogenase complex dissociates into the separate nitrogenase proteins: this is the rate-limiting step of the cycle [8]. Subsequently the Fe protein is reduced first by the present reductant, after which MgADP is rapidly replaced by MgATP, to complete the Fe protein cycle. For the reduction of N_2 to 2 NH_3 and H_2 (the inevitable side product of the nitrogenase reaction), the MoFe protein must be stepwise reduced in eight consecutive Fe protein cycles: this is described in the MoFe protein cycle [6].

In order to explain and to be able to simulate the observation that the measured specific H_2 production activity of the *Klebsiella pneumoniae* Fe protein, Kp2, is only 45% of the calculated maximum specific activity, Thorneley and Lowe assumed that only 45% of all Kp2 present is active [9]. The remaining 55% of total Kp2 is considered to be inactive with respect to electron transfer to Kp1, but is still capable of binding to Kp1 (with lower rate constants for binding to and dissociation from Kp1 than active Kp2 [9]). This assumption also explains the observation that a ratio [Kp2]/[Kp1] $\geq$ 4.5 is needed to obtain maximum electron transfer from the Fe protein to the MoFe protein: if all Kp2 would be active a ratio [Kp2]/[Kp1] = 2 would be sufficient [10].

A close structural similarity between the nucleotide binding sites of Av2 and the H-*ras* p21 protein was found [2,5]. A comparison between the mechanism of MgATP hydrolysis dependent electron transfer by nitrogenase and the mechanisms of action of molecular switch proteins like the GTPases and the muscle protein myosin was made. Like these proteins nitrogenase could be using nucleotide binding and hydrolysis as a kinetic mechanism in order to switch between different conformations of the protein.

Aluminium fluoride acts as an analogue of phosphate for various GTPases and ATPases, when GDP/ADP is present [11]. The crystal structures of transducin α complexed with MgGDP and aluminium fluoride (1.7 Å resolution) [12], myosin II (*Dictyostelium discoideum*) complexed with MgADP and aluminium fluoride (2.6 Å) [13] and the α-subunit of the G protein G_{i1} complexed with MgGDP and aluminium fluoride (2.2 Å) [14], revealed that in all of these cases GDP/ADP·aluminium fluoride is a transition state analogue of GTP/ATP, rather than an analogue of either the state before (GTP/ATP) or after hydrolysis (GDP/ADP·P_i) of GTP/ ATP. Aluminium fluoride occupies the position normally filled by the γ-phosphate of GTP/ATP. In contrast to the tetrahedral geometry of a phosphate group the aluminium is octahedrally coordinated with four fluoride ions (in the equatorial plane) and one of the oxygens of the β-phosphate and presumably one oxygen from a water molecule as the axial ligands. This structure resembles the pentacoordinated structure which the γ- phosphate transiently adopts during the transition state of the hydrolysis of GTP/ATP [11–14].

In this paper it is shown that aluminium fluoride together with MgADP inhibits nitrogenase from *A. vinelandii* by stabilizing the normally transient protein complex, which is temporary formed during the Fe protein cycle. The stability and the composition of the nitrogenase·ADP·aluminium fluoride complex were investigated.

2. Materials and methods

Azotobacter vinelandii ATCC strain 478 was grown and the nitrogenase component proteins were purified as described elsewhere [7]. Pro-

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Abbreviations: Av1 and Av2: MoFe protein and Fe protein from *Azotobacter vinelandii* nitrogenase, respectively; Kp1 and Kp2: MoFe protein and Fe protein from *Klebsiella pneumoniae* nitrogenase, respectively.

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tein concentrations were estimated by the microbiuret method after a precipitation step with deoxycholic acid and trichloric acid [7]. The molar concentrations of Av1 and Av2 were calculated from their molecular masses, 230 kDa and 63 kDa, respectively. Av1 contained 1.8 ± 0.2 Mo/mol Av1; the iron content of the Fe protein was 3.6 ± 0.3 mol Fe/mol Av2 [7].

The acetylene reduction activity (at 30°C) of the nitrogenase proteins was determined as described elsewhere [7]. The specific activity of each of the nitrogenase proteins was calculated from the maximum acetylene reduction rate, which was obtained at an optimum [Av2]/[Av1] ratio. Thus, for measurement of the specific activity of Av1, a 10-fold amount of Av2 was added to the reaction mixture. For measurement of the specific activity of Av2, the ratio [Av1]/[Av2] was varied at constant [Av2]; the maximum acetylene reduction rate obtained from this titration (usually at [Av1]/[Av2] ≈ 1.5) yielded the specific activity of Av2. The specific activities of Av1 and Av2 were at least 8 mol ethylene produced · s^{-1} · mol $Av1^{-1}$ and 2 mol ethylene produced · s^{-1} · mol $Av2^{-1}$, respectively. To investigate the effect of MgADP and aluminium fluoride on the activity of nitrogenase, 1 mM ADP, 1.5 mM $MgCl_2$, 0.7 mM aluminium fluoride ($AlF_3.H_2O$) and 7.0 mM KF (in order to dissolve $AlF_3.H_2O$ and obtain AlF_4^- the presence of excess F^- is required) was added to the incubation mixture; extra P_i (20 mM) was added during the activity measurements.

The ATPase activity of the nitrogenase complex as a function of the ratio [Av2]/[Av1] (Fig. 1) was determined under the same conditions as the measurement of the Av1 activity. Samples were taken from the reaction mixture and assayed for creatine by a slightly modified version of the method of Ennor [15].

For the measurements of electron transfer from Av2 to Av1 a HI-TECH SF-51 stopped-flow spectrophotometer (Salisbury, Wilts, UK), equipped with an anaerobic kit and a data acquisition and analysis system, was used. The absorbance changes at 430 nm were measured. One syringe of the stopped-flow apparatus contained 20 μM Av1 and Av2 in the indicated concentration. To investigate the effect of ADP and aluminium fluoride on the electron transfer 1 mM AlF_4^- (+10 mM KF), 1 mM ADP or both were added to this solution. The other syringe contained 10 mM ATP. Both syringes contained 10 mM $MgCl_2$, 100 mM NaCl, 5 mM sodium dithionite and 50 mM TES/NaOH, pH 7.4. The reaction temperature was 22.0 ± 0.1°C.

The amount of ADP bound in the (nitrogenase · ADP · aluminium fluoride) complex was measured after addition of $HClO_4$ (10%) to the complex. After neutralization with $KHCO_3$, ADP was converted into ATP using pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate. ATP was determined using a bioluminescence assay with luciferin and luciferase (EC 1.13.12.7). The intensity of the emitted light which was liberated by the luciferase reaction was directly proportional to the ATP concentration. An internal standard of ATP was employed to correct for inhibition of luciferase and for emission interference by compounds present in the incubation mixture.

The amounts of Av2 and Av1 present in the (nitrogenase · ADP · aluminium fluoride) complex were determined by a quantitative ELISA (Enzyme Linked ImmunoSorbent Assay) determination, using antibodies against Av1 and Av2, respectively. Purified Av1 and Av2 were used for calibration.

EPR spectra were obtained with a Bruker EPR-200 D spectrometer, with peripheral instrumentation and data acquisition as described elsewhere [16]. The concentrations in the EPR sample were: 9 mg protein/ml (nitrogenase · ADP · AlF_4^-) complex, 2 mM sodium dithionite and 500 mM NaCl in 50 mM TES/NaOH, pH 7.4. The EPR conditions for the measurements of the FeMoco $S = 3/2$ signal and the signals of oxidized P-clusters were described by Pierik et al. [16] and the EPR conditions for the measurements of the Av2 $S = 1/2$ and $S = 3/2$ signals were described by Hagen et al. [17].

All buffers used were saturated with argon. ATP (special quality) and ADP, creatine kinase, creatine phosphate and the ATP bioluminescence assay kit were obtained from Boehringer, aluminium fluoride monohydrate ($AlF_3.H_2O$) from Janssen Chimica.

3. Results and discussion

3.1. Inhibition of nitrogenase activity by MgADP and aluminium fluoride

The effect of AlF_4^- and MgADP on the nitrogenase activity was investigated, see Table 1. After 5 minutes incubation of the nitrogenase proteins (as indicated) the activity was measured.

Incubation of the nitrogenase proteins ([Av2]/[Av1] = 3) with MgADP or P_i did not affect the activity of Av1 and Av2. When the nitrogenase proteins were incubated with both MgADP and AlF_4^-, acetylene reduction was almost completely inhibited. Addition of P_i together with AlF_4^- to the nitrogenase proteins prevented the inhibition of the activity by MgADP and AlF_4^-. This indicates that P_i and AlF_4^- bind at the same position to nitrogenase. When P_i was added after 5 minutes incubation of the nitrogenase proteins with MgADP and AlF_4^-, acetylene reduction was still almost completely inhibited, which shows that P_i cannot exchange with AlF_4^- once MgADP and AlF_4^- are bound to the nitrogenase complex.

Also when excess Av2 ([Av2]/[Av1] = 10) was present during incubation with MgADP and AlF_4^-, acetylene reduction was completely inhibited (data not shown). When after the incubation extra Av2 (8.0 μM) and P_i (20 mM) were added, also no activity was observed, indicating that all Av1 was present in the inhibited nitrogenase · ADP · AlF_4^- complex. It was checked if Av2 was not inactivated during the incubation with MgADP and AlF_4^-. When after the incubation of the nitrogenase proteins ([Av2]/[Av1] = 10) with MgADP and AlF_4^- the original amount of Av1 (0.8 μM) and P_i (20 mM) were added, nitrogenase activity was observed. This activity corresponded to the nitrogenase activity normally found when [Av2]/[Av1] = 5. This indicates that the free Av2 (not bound in the nitrogenase · ADP · AlF_4^- complex) was not inhibited by MgADP and AlF_4^- during the incubation.

Stopped-flow measurements showed that incubation of the nitrogenase proteins with only AlF_4^- before mixing with MgATP did not affect the pre-steady-state MgATP-induced electron transfer reaction. This indicates that AlF_4^- does not rapidly bind and inhibit nitrogenase before MgATP hydrolysis.

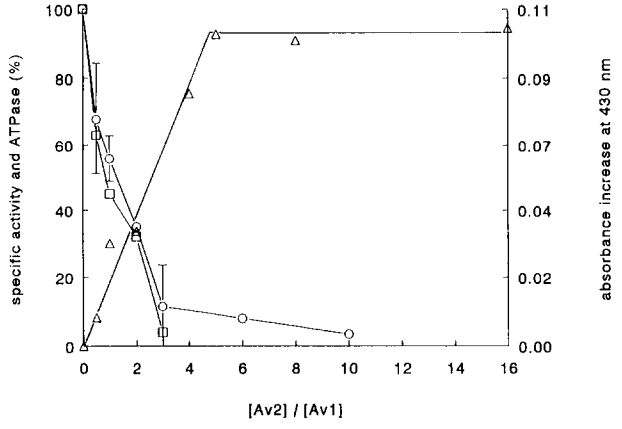

Fig. 1. Pre-steady-state electron transfer (ΔA_{430}) as a function of the ratio [Av2]/[Av1], and the inhibition of the specific activity of Av1 and of the ATPase activity by aluminium fluoride and MgADP as a function of [Av2]/[Av1]. Electron transfer (△) was measured in the absence of MgADP and aluminium fluoride. The concentration Av1 after mixing was 10 μM. The acetylene reduction activity (○) and ATPase activity (□) were measured after incubation (at room temperature) of the nitrogenase proteins with aluminium fluoride and MgADP. The concentration Av1 was 0.8 μM. The error bars on the acetylene reduction data points show the deviation from the average of three measurements. In the absence of aluminium fluoride and P_i the 100% activity was 6.9 mol C_2H_4 produced · s^{-1} · mol $Av1^{-1}$ and the 100% ATPase activity was 50.4 mol ADP produced · s^{-1} · mol $Av1^{-1}$.

When the nitrogenase proteins were incubated with both MgADP and AlF_4^-, no electron transfer was observed after mixing with MgATP (data not shown).

The results show that AlF_4^- and MgADP together inhibit the overall nitrogenase activity and the pre-steady-state electron transfer reaction. Addition of MgATP or P_i does not reverse the inhibition. Since both Av2 and Av1 are involved in the inhibition, it seems likely that AlF_4^- and MgADP stabilize a normally transient nitrogenase complex; for catalysis association of Av2 and Av1 and dissociation of the nitrogenase complex are required (Fe protein cycle) [6,8].

3.2. *Inhibition of nitrogenase activity as a function of [Av2]/[Av1]*

In Fig. 1 the amplitude of the absorbance increase (430 nm) associated with the MgATP-induced pre-steady-state electron transfer reaction from Av2 to Av1 is shown as a function of the ratio [Av2]/[Av1]. No MgADP and AlF_4^- were present in the reaction mixture. The absorbance amplitude ($\Delta A_{430} = 0.102$) was maximal when [Av2]/[Av1] ≥ 4.5. Similar data were observed for *K. pneumoniae* nitrogenase by Ashby and Thorneley [10], and were explained by the assumption that only 45% of the Fe protein is active with respect to electron transfer [9]. The observed rate constant of electron transfer was independent of the ratio [Av2]/[Av1]: $k_{obs} \approx 120\ s^{-1}$ (data not shown).

In the same figure the acetylene reduction activity and the ATPase activity of the nitrogenase complex in the presence of AlF_4^- and MgADP at various [Av2]/[Av1] are shown. After 20 minutes incubation of the nitrogenase proteins with MgADP and AlF_4^-, excess Av2 (10-fold [Av1]) and P_i (20 mM) were added to the incubation mixture and the activity was measured. No difference was observed between the inhibition of the activity after 10 minutes or 20 minutes incubation at low ratios ([Av2]/[Av1] ≤ 2); at higher ratios an incubation for 5 minutes already yielded the same inhibition as 20 minutes incubation (data not shown). The 100% activity was determined in an incubation without AlF_4^- and P_i: 6.9 mol ethylene produced · s^{-1} · mol $Av1^{-1}$. Complete inhibition of the specific activity was observed when [Av2]/[Av1] ≥ 3 during the incubation of the samples with AlF_4^- and MgADP. At lower ratios apparently not all Av1 could be bound in the inhibited nitrogenase · ADP · AlF_4^- complex.

The ATPase activity of the nitrogenase complex was inhibited by the presence of AlF_4^- and MgADP and follows the same inhibition pattern as the acetylene reduction activity: at ratio [Av2]/[Av1] ≥ 3 MgATP hydrolysis hardly takes place any more, see Fig. 1.

Table 1
Inhibition of the acetylene reduction activity of nitrogenase by aluminium fluoride and MgADP

Exp.	Additions	Specific activity (%)
1	–	100
2	P_i	100
3	AlF_4^-	2
4	AlF_4^- + P_i	100
5	AlF_4^-, after 5′: P_i	3

The nitrogenase proteins ([Av1] = 0.8 μM; [Av2] = 2.4 μM) were incubated with ADP (1 mM), $MgCl_2$ (1.5 mM), sodium dithionite (16 mM) and TES/NaOH (50 mM), pH 7.4, at room temperature for 5 min. If present in the incubation mixture: [AlF_4^-] = 0.7 mM (plus 7 mM KF) and [P_i] = 20 mM. The acetylene reduction activity (at 30°C) of Av1 was measured without addition of extra Av2. In the absence of aluminium fluoride, at a ratio [Av2]/[Av1] = 3, the activity was 100% = 3.1 mol C_2H_4 produced · s^{-1} · mol $Av1^{-1}$.

Table 2
Recovery of the acetylene reduction activity after separation (on DEAE cellulose) of the ADP-aluminium fluoride bound nitrogenase complex from free Av2, MgADP and AlF_4^-

Exp.	Incubation	Specific activity (%)
1		2
2	+ P_i + $MgCl_2$ + NaCl	0
	5 min 50°C	7
3	overnight, room temperature,	
	+ P_i + $MgCl_2$ + NaCl	14
	45 min 50°C	69
4	+ P_i + $MgCl_2$ + NaCl	2
	120 min 50°C	46

In experiments 2, 3 and 4: [P_i] = 20 mM, [$MgCl_2$] = 10 mM and [NaCl] = 0.64 M. After incubation the specific activity of Av1 was determined. The specific activity of Av1 in the absence of MgADP and AlF_4^- was 6.9 mol C_2H_4 produced · s^{-1} · mol $Av1^{-1}$ = 100%.

3.3. *Isolation of the nitrogenase · ADP · AlF_4^- complex*

The nitrogenase proteins ([Av2]/[Av1] = 6) were incubated with MgADP and AlF_4^- for 10 minutes at room temperature, to form the inhibited nitrogenase · ADP · AlF_4^- complex. The protein complex was bound to DEAE cellulose and free MgADP and AlF_4^- were removed by an 80 mM NaCl (plus 2 mM sodium dithionite in 50 mM TES/NaOH, pH 7.4) wash procedure. At 300 mM NaCl the nitrogenase · ADP · AlF_4^- complex eluted (which was verified by an ELISA determination) and at 500 mM NaCl free Av2 came off the column. The activity of Av1 in the nitrogenase complex after this treatment was 2% of the specific activity in the absence of MgADP and AlF_4^- (6.9 mol ethylene produced · s^{-1} · mol $Av1^{-1}$). The specific activity of the free Av2 was 2.0 mol ethylene produced · s^{-1} · mol $Av2^{-1}$, which is as high as the specific activity of Av2 before formation of the nitrogenase · ADP · AlF_4^- complex. If the assumption that inactive Av2 binds to Av1 with a 5-fold higher dissociation constant than active Av2 is correct [9], more active than inactive Av2 would be expected to be bound in the nitrogenase · ADP · AlF_4^- complex, and the specific activity of the free Av2 would be expected to be lower than the obtained value. We conclude that for the formation of the nitrogenase · ADP · AlF_4^- complex all Av2 is equally active.

After preparation of the inhibited nitrogenase · ADP · AlF_4^- complex, attempts were made to reactivate the protein complex and to measure the activity of the nitrogenase proteins. The results are given in Table 2.

Addition of $MgCl_2$ (10 mM), P_i (20 mM) and NaCl (0.64 mM) to the nitrogenase · ADP · AlF_4^- complex did not recover the activity of Av1. After incubation of the complex for 5 minutes at 50°C (in the presence of $MgCl_2$, P_i and NaCl) some of the Av1 activity was restored: 7% (Table 2, exp. 2).

Incubation of the nitrogenase · ADP · AlF_4^- complex overnight (under argon) at room temperature in the presence of $MgCl_2$, P_i and NaCl, increased the specific activity of Av1 to 14%. This shows that some dissociation of the nitrogenase · ADP · AlF_4^- complex occurs in time. Hereafter the mixture was incubated at 50 °C: after 45 minutes, 69% of the specific activity of Av1 was recovered (Table 2, exp. 3).

When, immediately after preparation (as described above),

the inhibited nitrogenase·ADP·AlF_4^- complex was incubated at 50°C (in the presence of $MgCl_2$, P_i and NaCl), the specific activity of Av1 increased to 46% in 2 hours (Table 2, exp. 4). Measurement of the nitrogenase activity without adding extra Av2, showed that the Av2 released from the inhibited complex also still has activity: 0.6 mol ethylene produced·s^{-1}·mol $Av2^{-1}$. Considering that in the incubation mixture the ratio [Av2]/[Av1] will be about 2; and that under these conditions the activity of Av2 is only 50% of its maximum specific activity [18], the activity found for Av2 is in good agreement with the 46% specific activity found for Av1 after dissociation from the inhibited complex (Table 2, exp. 4).

The results show that the inhibited nitrogenase·ADP·AlF_4^- complex is rather stable: after 20 hours only 14% of the complex is dissociated. The nitrogenase proteins are not inactivated by the association with MgADP and AlF_4^-: the specific activity of both nitrogenase proteins recovers in time by incubation of the complex at 50 °C.

3.4. Characterization of the nitrogenase·ADP·ALF_4^- complex

The amounts of Av2, Av1 and ADP in the nitrogenase·ADP·AlF_4^- complex were determined: 2.7 ± 0.2 Av2/Av1 and 2.0 ± 0.2 ADP/Av1 were present. Cordewener et al. [19] observed that two molecules of MgADP bind to Av2, but that only one MgADP molecule binds very tightly to Av2. The less tightly bound MgADP apparently is not present in the nitrogenase·ADP·AlF_4^- complex.

EPR measurements (data not shown) of the nitrogenase·ADP·AlF_4^- complex did not show the $S = 1/2$ and $S = 3/2$ signals of reduced Av2, indicating that Av2 was oxidized in the complex. The FeMoco $S = 3/2$ signal was fully present, comparable to the FeMoco signal of dithionite- reduced Av1. A yet unidentified signal at $g = 5.3$ was observed. This was not a signal associated with partly oxidized P-clusters as observed by Pierik et al. [16] or Tittsworth et al. [20]. It is unlikely that an electron was transferred from Av2 to the P- cluster, since all eight Fe atoms of the P-cluster are thought to be in the ferrous state [21]. A possibility might be that in the nitrogenase·ADP·AlF_4^- complex the redox potential of the [4Fe–4S] cluster of Av2 is so low that at the ambient redox potential (about −550 mV) HSO_3^-, present in a sodium dithionite solution, is reduced by Av2 to SO_2^-.

The inhibition of nitrogenase activity by AlF_4^- together with MgADP resembles the action of AlF_4^- together with GDP/ADP on the activity of molecular switch proteins. In analogy to these proteins it is possible that a transition state nitrogenase complex is formed. An important difference with the molecular switch proteins however is that both nitrogenase proteins are necessary for complex formation with AlF_4^- and MgADP, whereas in case of the molecular switch proteins a stable complex is formed from MgGDP/MgADP, AlF_4^- and only the nucleotide binding protein [11–14]. Since the transition state complex is very stable, it would be worthwhile trying to crystallize the nitrogenase·ADP·AlF_4^- complex and determine its three-dimensional structure.

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References

[1] Hardy, R.W.F. and Burns, R.C. (1968) Annu. Rev. Biochem. 37, 331–358.
[2] Howard, J.B. and Rees, D.C. (1994) Annu. Rev. Biochem. 63, 235–264.
[3] Hagen, W.R., Wassink, H., Eady, R.R., Smith, B.E. and Haaker, H. (1987) Eur. J. Biochem. 169, 457–465.
[4] Kim, J. and Rees, D.C. (1992) Science 257, 1677–1682.
[5] Georgiadis, M.M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J.J. and Rees, D.C. (1992) Science 257, 1653–1659.
[6] Lowe, D.J. and Thorneley, R.N.F. (1984) Biochem. J. 224, 877–886.
[7] Duyvis, M.G., Wassink, H. and Haaker, H. (1994) Eur. J. Biochem. 225, 881–890.
[8] Thorneley, R.N.F. and Lowe, D.J. (1983) Biochem. J. 215, 393–403.
[9] Thorneley, R.N.F. and Lowe, D.J. (1984) Biochem. J. 224, 903–909.
[10] Ashby, G.A. and Thorneley, R.N.F. (1987) Biochem. J. 246, 455–465.
[11] Chabre, M. (1990) Trebds Biochem. Sci 15, 6–10.
[12] Sondek, J., Lambright, D.G., Noel, J.P., Hamm, H.E. and Sigler, P.B. (1994) Nature 372, 276–279.
[13] Fisher, A.J., Smith, C.A., Thoden, J., Smith, R., Sutoh, K., Holden, H.M. and Rayment, I. (1995) Biophys. J. 68, 19s-28s.
[14] Coleman, D.E., Berghuis, A.M., Lee, E., Linder, M.E., Gilman, A.G. and Sprang, S.R. (1994) Science 265, 1405–1412.
[15] Ennor, A.H. (1957) Methods Enzymol. 3, 850–861.
[16] Pierik, A.J., Wassink, H., Haaker, H. and Hagen, W.R. (1993) Eur. J. Biochem. 212, 51–61.
[17] Hagen, W.R., Eady, R.R., Dunham, W.R. and Haaker, H. (1985) FEBS Lett. 189, 250–254.
[18] Cordewener, J., Krüse-Wolters, M., Wassink, H., Haaker, H. and Veeger, C. (1988) Eur. J. Biochem. 172, 739–745.
[19] Cordewener, J., ten Asbroek, A., Wassink, H., Eady, R., Haaker, H. and Veeger, C. (1987) Eur. J. Biochem. 162, 265–270.
[20] Tittsworth, R.C. and Hales, B.J. (1993) J. Am. Chem. Soc. 115, 9763–9767.
[21] Surerus, K.K., Hendrich, M.P., Christie, P.D., Rottgardt, D., Orme-Johnson, W.H. and Münck, E. (1992) J. Am. Chem. Soc. 114, 8579–8590.