

Thermodynamics of the mechanism of the nitrogenase reaction

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Received 3 November 2004; accepted 16 November 2004

Available online 10 December 2004

Abstract

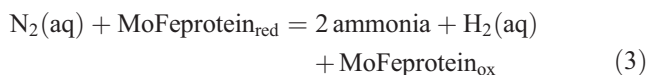
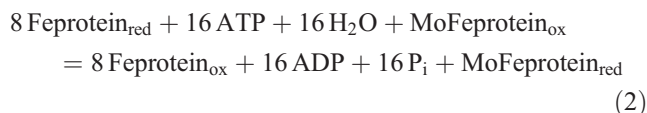
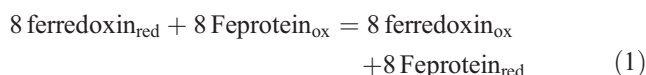
The fixation of molecular nitrogen by nitrogenase requires a lot of energy because 16 mol of ATP are hydrolyzed per mole of nitrogen converted to ammonia. Kim and Dees determined the crystallographic structure of nitrogenase and this has led to a three-step mechanism that involves Feprotein and MoFeprotein in addition to ferredoxin. Each of these steps can be interpreted in terms of two half reactions that are connected through their transfer of electrons. Estimates can be made of the standard apparent reduction potentials of these three steps and their dependencies on pH and ionic strength. This mechanism is compared with the same type of analysis of an alternative three-step mechanism in which the hydrolysis of ATP is coupled with the reduction of molecular nitrogen, rather than the reduction of Feprotein. The problem with the first mechanism is that the second step produces 12 mol of hydrogen ions per mole of nitrogen fixed and the third step consumes 10 mol of hydrogen ions per mole of nitrogen fixed. The alternative mechanism does not have this problem.

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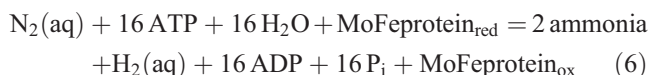
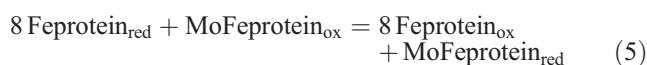
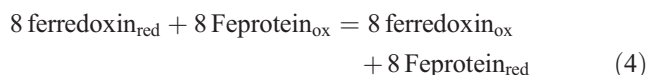
Keywords: Nitrogenase; Apparent reduction potentials; Mechanisms; Transformed Gibbs energy; Half reactions; Thermodynamics; Ferredoxin

1. Introduction

It is recognized that the enzyme-catalyzed fixation of molecular nitrogen to ammonia is an energetically demanding series of reactions. Burris [1] found that about 16 mol of ATP are hydrolyzed per mole of nitrogen fixed. It was suggested [2] that the role of the hydrolysis of ATP may be to supply hydrogen ions required by the reduction reaction so that the catalytic site does not become too alkaline. At pH 7, about 10 mol of hydrogen ions are required by the reaction $\text{N}_2(\text{aq}) + 8 \text{ ferredoxin}_{\text{red}} = 2 \text{ ammonia} + \text{H}_2(\text{aq}) + 8 \text{ ferredoxin}_{\text{ox}}$ because nitrogenase produces a mole of molecular hydrogen per mole of molecular nitrogen fixed. Kim and Dees [3,4] determined the crystallographic structure of the catalytic site of nitrogenase from *Azotobacter vinelandii*. Their description of the mechanism has led to the following three reactions as the steps in the nitrogenase reaction at a specified pH [5]:



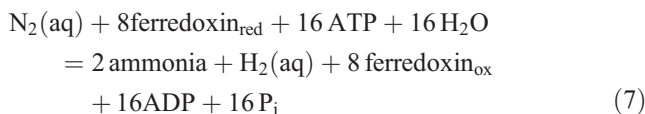
In this article, the thermodynamics of reactions (1)–(3) and their half reactions are discussed and compared with an alternative three-step mechanism:



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This alternative mechanism moves the contribution of the hydrolysis of ATP from the second step to the third step. These three steps add up to



which is the same net reaction that is obtained by adding reactions (1)–(3).

Since these seven reactions are redox reactions, they can each be discussed in terms of half reactions and standard apparent reduction potentials E° . In the nitrogenase literature, the term standard reduction potential and the symbol E° have often been used, but the term standard apparent reduction potential and symbol E° should be used at specified pH (and sometimes specified $p\text{Mg}$). The reason is that the term standard reduction potential and symbol E° are used in chemistry where the standard state of the hydrogen ion is unit activity in water at each temperature. When the pH is specified, the adjective “apparent” should be used because of the dependence on pH. The criterion for spontaneous change and equilibrium at specified pH is provided by the transformed Gibbs energy G' that is defined by a Legendre transform [6]. This leads to standard transformed Gibbs energies of formation $\Delta_f G'^\circ$, standard transformed reaction Gibbs energies $\Delta_r G'^\circ$, apparent equilibrium constants K' , and standard apparent reduction potentials [7]. E° for a half reaction can be calculated from the standard transformed reaction Gibbs energy for the half reaction by using

$$\Delta_r G'^\circ = -\nu F E^\circ \quad (8)$$

where ν is the number of formal electrons transferred and F is the faraday (96,485 C mol⁻¹). In making calculations of standard apparent reduction potentials, the half reactions are written as reduction reactions. The standard transformed Gibbs energy for a half reaction can be calculated from the standard transformed Gibbs energies of formation $\Delta_f G'^\circ$ of the reactants in a half reaction using

$$\Delta_r G'^\circ = \sum \nu_i \Delta_f G'^\circ_i \quad (9)$$

where ν_i is the stoichiometric number for a reactant in the half reaction. The $\Delta_f G'^\circ$ of the reactants can be calculated if the standard Gibbs energies of formation $\Delta_f G_j^\circ$ are known for all the species involved at the specified pH [7–9]. When Eq. (9) is applied to a full reaction, the apparent equilibrium constant K' can be calculated using

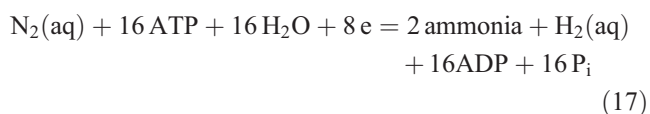
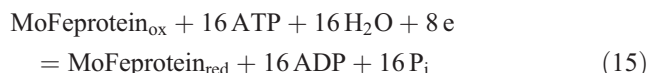
$$\Delta_r G'^\circ = -RT \ln K' \quad (10)$$

and

$$K' = \exp(\nu F E^\circ / RT) \quad (11)$$

2. Discussion in terms of half reactions

Reactions (1)–(7) can each be divided into two half reactions, and there are six different half reactions:



Half reactions can be multiplied by integers without changing their standard apparent reduction potentials. If the standard apparent reduction potentials of these six half reactions can be calculated, they can be used to calculate the apparent equilibrium constants of reactions (1)–(7). The standard Gibbs energies of formation of all the species in half reactions (12), (16), and (17) are known and are available in BasicBiochemData2 [10]. These data are stored in small matrices in Mathematica [11] with a row for each species. The first entry in a row in the data matrix is $\Delta_f G'^\circ$, the second is $\Delta_f H'^\circ$, the third is the electric charge, and the fourth is the number of hydrogen atoms in the species. The properties are for 298.15 K and zero ionic strength, and the energies of formation are in kJ mol⁻¹.

Stombaugh et al. [12] measured the standard apparent reduction potentials for a number of ferredoxins at 298.15 K and 0.25 M ionic strength and found that they varied from −0.377 to −0.434 V at pH 7. The calculations presented here are based on their value of −0.403 V for ferredoxin from *Claustidium pasteurianum*. Since Tagawa and Arnon [13] found that the reduction potential for ferredoxin is independent of pH in the range pH 6.1–7.4, it is assumed that the redox site of ferredoxin does not involve any pKs in the region pH 5 to 9. Since ferredoxin is a protein, the thermodynamic properties apply to the reactive site. $\Delta_f G'^\circ$ of the ferredoxinoxsp site is arbitrarily assigned the value zero, much like $\Delta_f G'^\circ(\text{H}^+)$ is assigned the value zero at zero ionic strength in tables of chemical thermodynamic properties. The 38.07 kJ mol⁻¹ in Table 1 comes from $-(96.485 \text{ kC mol}^{-1})(-0.403 \text{ V})$.

The standard apparent reduction potentials of half reactions (13) and (14) have not been determined experimentally, but we are not completely in the dark about E° (half reaction (13)) and E° (half reaction (14)) because for reaction (4) to produce significant concentrations of Feprotein_{red}, the apparent equilibrium constant for this

Table 1

Thermodynamic properties of redox sites in proteins at 298.15 K and zero ionic strength (The charge number is z , and the number of hydrogen atoms is N_H)

	$\Delta_f G^\circ / \text{kJ mol}^{-1}$	z	N_H
ferredoxinoxsp	0	1	0
ferredoxinredsp	38.07	0	0
Feproproteinoxsp	0	1	0
Feproproteinredsp	38.07	0	0
MoFeproproteinoxsp	0	0	0
MoFeproproteinredsp	304.56	0	0

reaction needs to be unity or larger. In order for reaction (5) to produce a significant concentrations of MoFeproprotein_{red}, the apparent equilibrium constant for this reaction needs to be unity or larger. Another way to describe the situation is to say that the standard apparent reduction potentials of ferredoxin, Feproprotein, and MoFeproprotein must be about the same. If they are the same, the apparent equilibrium constants for reactions (1), (4), and (5) will each be unity at zero ionic strength. The following calculations are based on $E^\circ(\text{ferredoxin}) = E^\circ(\text{Feproprotein}) = E^\circ(\text{MoFeproprotein})$ at zero ionic strength because this is a kind of minimal condition. In order for $E^\circ(\text{MoFeproprotein})$ to be equal to $E^\circ(\text{Feproprotein})$,

$$\begin{aligned} & -\Delta_f G^\circ(\text{MoFeproproteinredsp})/8F \\ &= -\Delta_f G^\circ(\text{Feproproteinredsp})/F \end{aligned} \quad (18)$$

Since these three proteins have oxidized and reduced forms, the species are distinguished by adding red and ox to the names of the proteins. Thus, $\Delta_f G^\circ(\text{MoFeproproteinredsp}) = 8 \times 38.07 \text{ kJ mol}^{-1} = 304.56 \text{ kJ mol}^{-1}$ at 298.15 K and zero ionic strength. The species properties of the redox sites in the proteins involved are given in Table 1.

Strictly speaking, the charge on MoFeproprotein_{oxsp} should be taken as 8+. However, it is considered to be unreasonable to have this large a charge at a point in the Debye–Huckel adjustment for ionic strength, and so no ionic strength adjustment is made for MoFeproprotein_{oxsp}. This large charge is undoubtedly spread over the structure of the MoFe site. It is assumed that no pKs in Feproprotein and MoFeproprotein have to be taken into account.

The function of pH and ionic strength that gives $\Delta_f G^\circ$ for these three reactants (sums of species) can be derived by use of the Mathematica program calcdGmat [10,14]. These functions make it possible to calculate $\Delta_f G^\circ$, $\Delta_r N_H$, and E° for half reactions (12)–(17) and $\Delta_f G^\circ$, $\Delta_r N_H$, E° , and K' for biochemical reactions (1)–(7). Half reactions (12)–(17) pass on their standard transformed thermodynamic properties to full reactions (1)–(7).

3. Calculation of standard transformed thermodynamic properties of half reactions (12)–(17)

The properties of half reactions (12)–(17) are calculated using the Mathematica program derivfnGNHEMFrX that is

given in Appendix A. This program derives the functions of pH and ionic strength that give the standard transformed Gibbs energy of reaction, change in number of hydrogen ions bound, and the standard apparent reduction potential at 298.15 K for a biochemical half reaction. When a half reaction is typed in and the number of formal electrons is given, this program yields a list of the three mathematical functions of pH and ionic strength. The change in the binding of hydrogen ions in a half reaction or a full reaction is calculated using

$$\Delta_r N_H = -\frac{\nu F}{RT \ln(10)} \left(\frac{\partial E'^\circ}{\partial \text{pH}} \right)_{T,P} \quad (19)$$

The half reactions in this table are listed in order of decreasing standard reduction potentials at pH 7. Tables like this are very useful in studying redox reactions because half reactions higher in the table can drive half reactions lower in the table. The half reactions involving molecular oxygen and molecular hydrogen are included because they define the boundaries for standard apparent reduction potentials in aqueous solutions. Half reactions with E° more positive than 0.807 V at 298.15 K, pH 7, and ionic strength 0.25 M can liberate oxygen gas at more than 1 bar. Half reactions with E° more negative than -0.423 V at 298.15 K, pH 7, and ionic strength 0.25 M can liberate hydrogen gas at more than 1 bar. The half reactions involving molecular hydrogen are written without hydrogen ions because it is understood that in these biochemical half reactions, hydrogen ions are supplied or removed to maintain the pH at the specified value. The E° for chemical reactions are not functions of the pH. The $\Delta_r N_H$ are given in Table 2 for each half reaction. When the difference is taken between two half reactions, the change in binding of hydrogen ions in the full reaction is given by

$$\Delta_r N_H = \Delta_r N_H(\text{h}) - \Delta_r N_H(\text{l}) \quad (20)$$

$\Delta_r N_H(\text{h})$ is for the half reaction with the higher E° and is for the half reaction with the lower E° .

4. Calculation of standard thermodynamic properties of biochemical reactions (1)–(7)

In Mathematica, $\Delta_f G^\circ$, $\Delta_r N_H$, and K' for reactions (1)–(7) are calculated using the Mathematica program derivfnGNHKprimerX that is given in Appendix A. This program is used to calculate $\Delta_f G^\circ$ in kJ mol^{-1} and $\Delta_r N_H$ reactions (1)–(7). The results are tabulated in Table 3.

Note that for reaction (2), the apparent equilibrium constant increases very rapidly with pH because of the very large production of hydrogen ions in this reaction. For reaction (3), the apparent equilibrium constant decreases

Table 2

Standard apparent reduction potentials of half reactions in volts at 298.15 K, 0.25 M ionic strength, and three pHs

Half reaction		pH 6	pH 7	pH 8
$\text{O}_2(\text{aq}) + 4\text{e} = 2\text{H}_2\text{O}$	E°	0.908	0.849	0.790
	$\Delta_r N_{\text{H}}$	4	4	4
$\text{O}_2(\text{g}) + 4\text{e} = 2\text{H}_2\text{O}$	E°	0.866	0.807	0.747
	$\Delta_r N_{\text{H}}$	4	4	4
$13.\text{N}_2(\text{aq}) + 16\text{ATP} + 16\text{H}_2\text{O} + 8\text{e}$	E°	0.443	0.427	0.459
$= 2\text{ammonia} + \text{H}_2(\text{aq}) + 16\text{ADP} + 16\text{P}_i$	$\Delta_r N_{\text{H}}$	0.602	−1.88	−5.54
$11.\text{MoFeprotein}_{\text{ox}} + 16\text{ATP} + 16\text{H}_2\text{O} + 8\text{e}$	E°	0.294	0.352	0.457
$= \text{MoFeprotein}_{\text{red}} + 16\text{ADP} + 16\text{P}_i$	$\Delta_r N_{\text{H}}$	−3.98	−11.97	−15.43
$12.\text{N}_2(\text{aq}) + 8\text{e} = 2\text{ammonia} + \text{H}_2(\text{aq})$	E°	−0.245	−0.319	−0.393
	$\Delta_r N_{\text{H}}$	10.00	9.99	9.89
$10.\text{MoFeprotein}_{\text{ox}} + 8\text{e} = \text{MoFeprotein}_{\text{red}}$	E°	−0.395	−0.395	−0.395
	$\Delta_r N_{\text{H}}$	0	0	0
$8.\text{ferredoxin}_{\text{ox}} + 8\text{e} = 8\text{ferredoxin}_{\text{red}}$	E°	−0.403	−0.403	−0.403
	$\Delta_r N_{\text{H}}$	0	0	0
$9.\text{Feprotein}_{\text{ox}} + \text{e} = \text{Feprotein}_{\text{red}}$	E°	−0.403	−0.403	−0.403
	$\Delta_r N_{\text{H}}$	0	0	0
$2\text{e} = \text{H}_2(\text{g})$	E°	−0.363	−0.423	−0.482
	$\Delta_r N_{\text{H}}$	2	2	2
$2\text{e} = 2\text{H}_2(\text{aq})$	E°	−0.455	−0.515	−0.573
	$\Delta_r N_{\text{H}}$	2	2	2

very rapidly with increasing pH because of the very large consumption of hydrogen ions in this reaction.

There are various ways, the entries in Tables 2 and 3 can be checked. The differences in the standard apparent reduction potentials in Table 2 must yield the standard transformed Gibbs energies of formation in Table 3. The differences in the changes in binding of hydrogen ion in Table 2 must yield the changes in the binding of hydrogen ions in Table 3. The changes in standard transformed reaction Gibbs energies with pH must agree with the changes in the binding of hydrogen ions in the reaction.

5. Discussion

In the mechanism with steps 1–3, the second step produces about 12 mol of hydrogen ions for each mole of $\text{MoFeprotein}_{\text{red}}$ produced. This step involves the transfer of eight electrons from Feprotein to MoFeprotein and the hydrolysis of 16 mol of ATP. The vicinity of this site will become very acidic. In the next step (reaction (3)), molecular nitrogen is fixed and $\text{MoFeprotein}_{\text{red}}$ gives up eight electrons. This third step requires 10 hydrogen ions from the solution in the vicinity, and so this region will

become very alkaline. The net reaction (reaction (7)) liberates 1.9 mol of hydrogen ions.

The alternative mechanism (reactions (4)–(6)) is quite different in the way hydrogen ions are produced and consumed. No hydrogen ions are produced or consumed in the second step (reaction (5)). The third step (reaction (6)) liberates 1.9 mol of hydrogen ions per mole of nitrogen fixed. Thus, the problem of the production of 12 mol of hydrogen ions at one site and consumption of 10 mol of hydrogen ions at another sites is avoided.

More positive values than $304.56 \text{ kJ mol}^{-1}$ could be used for $\Delta_r G^\circ(\text{mofeprotein}_{\text{red}})$, but that would make E° more negative than -0.423 V for the production for molecular hydrogen at one bar pressure at 298.15 K, pH 7, and ionic strength 0.25 M. Thus, it is not surprising that a mole of molecular hydrogen is produced by nitrogenase for each mole of nitrogen fixed. The E° for the proteins involved are about as low as they can be in aqueous solution without producing molecular hydrogen. Since the E° for the ferredoxins from various sources range from -0.377 to -0.434 V [12], more detailed calculations will have to be made for specific organisms.

The standard apparent reduction potentials for reactions involving phosphate esters and inorganic phosphate are

Table 3

Standard transformed Gibbs energies of reaction in kJ mol⁻¹ and changes in the binding of hydrogen ions at 298.15 K, 0.25 M ionic strength, and three pHs

Half reaction		pH 6	pH 7	pH 8
(1) 8ferredoxin _{red} + 8Feprotein _{ox}	$\Delta_r G^\circ$	0	0	0
= 8ferredoxin _{ox} + 8Feprotein _{red}	$\Delta_r N_H$	0	0	0
(2) 8Feprotein _{red} + 16ATP + 16H ₂ O + MoFeprotein _{ox}	$\Delta_r G^\circ$	-537	-583	-663
= 8Feprotein _{ox} + 16ADP + 16P _i + MoFeprotein _{red}	$\Delta_r N_H$	-4.0	-11.9	-15.4
(3) N ₂ (aq) + MoFeprotein _{red}	$\Delta_r G^\circ$	-115	-58	-1.4
= 2ammonia + H ₂ (aq) + MoFeprotein _{ox}	$\Delta_r N_H$	10.0	10.0	10.0
(4) 8ferredoxin _{red} + 8Feprotein _{ox}	$\Delta_r G^\circ$	0	0	0
= 8ferredoxin _{ox} + 8Feprotein _{red}	$\Delta_r N_H$	0	0	0
(5) 8Feprotein _{red} + MoFeprotein _{ox}	$\Delta_r G^\circ$	-6.5	-6.5	-6.5
= 8Feprotein _{ox} + MoFeprotein _{red}	$\Delta_r N_H$	0	0	0
(6) N ₂ (aq) + 16ATP + 16H ₂ O + 8ferredoxin _{red}	$\Delta_r G^\circ$	-647	-635	-659
= 2ammonia + H ₂ (aq) + 16ADP + 16P _i	$\Delta_r N_H$	6.0	-1.9	-5.5
+ 8ferredoxin _{ox}				
(7) N ₂ (aq) + 16ATP + 16H ₂ O + MoFeprotein _{red}	$\Delta_r G^\circ$	653	-641	-665
= 2ammonia + H ₂ (aq) + 16ADP + 16P _i	$\Delta_r N_H$	6.0	-1.9	-5.5
+ MoFeprotein _{ox}				

affected by magnesium ions, depending on $pMg = -\log[Mg^{2+}]$. The dissociation constants of the complex ions involved in the reactions discussed here are known [15] and have been used to calculate standard transformed thermodynamic properties for ATP+H₂O=ADP+P_i at temperatures of 298.15 and 313.15 K at pHs 5, 7, 9, and $pMgs$ 2, 4, 6 at 0.25 M ionic strength [16]. At 298.15 K, pH 7, and 0.25 M ionic strength, $\Delta_r G^\circ$ for the hydrolysis of ATP are -36.0, -35.0, and -30.8 kJ mol⁻¹ at pMg 2, 4, and 6. These adjustments can be applied to reactions (2), (6), and (7) because the binding of Mg²⁺ affects the thermodynamic properties of only ATP, ADP, and P_i.

The nitrogenase reaction is a remarkable example of coupling. About half of enzymes that have been assigned names couple two reactions and a few couple three reactions. The nitrogenase reaction has been discussed here in terms of coupling three steps, but some steps involve coupling large numbers of reactions.

Glossary

E°	standard reduction potential for a chemical reaction (V)
E°	standard apparent reduction potential for a reaction at specified pH (V)
F	faraday (96.485 kC mol ⁻¹)
$\Delta_r G^\circ$	standard Gibbs energy of formation of a species (kJ mol ⁻¹)
$\Delta_r H^\circ$	standard enthalpy of formation of a species (kJ mol ⁻¹)
$\Delta_r G^\circ$	standard transformed Gibbs energy of formation (kJ mol ⁻¹)
$\Delta_r G^\circ$	standard transformed reaction Gibbs energy of (kJ mol ⁻¹)

K'	apparent equilibrium constant at a specified pH (dimensionless)
N_H	number of hydrogen atoms in a species (dimensionless)
$\Delta_r N_H$	change in the number of hydrogen ions bound in a reaction at a specified pH (dimensionless)
R	gas constant (8.31451 J K ⁻¹ mol ⁻¹)
T	thermodynamic temperature (K)
ν	number of formal electrons transferred in a half reaction (dimensionless)
ν_i	stoichiometric number of a reactant in a reactions at specified pH (dimensionless)

Acknowledgements

I am indebted to Dr. Robert N. Goldberg (NIST) for many helpful discussions. Grateful thanks to NIH for support of this research by award number 5-RO1-GM48358-09.

Appendix A

The two following programs need functions of pH and ionic strength for each reactant. These functions can be obtained from BasicBiochemData2 for 131 reactants.

```

derivefnGNHEMFrX[eq_,nu_]:=Module[{function},
(*Derives the functions of pH and ionic strength that give the
standard transformed Gibbs energy of reaction, change in
number of hydrogen ions bound, and the standard apparent
reduction potential at 298.15 K for a biochemical half reaction
typed in as, for example, n2aq+de=2*ammonia+h2aq. nu is

```


the number of formal electrons. The standard transformed Gibbs energy of reaction is in kJ mol^{-1} .*)

```
function=Solve[eq,de];
functionG=function[[1,1,2]]; functionNH=(1/(8.31451*
0.29815*Log[10]))*D[functionG,pH];
functionEMF=-functionG/(nu*96.485);
{functionG,functionNH,functionEMF}
derivefnGNHKprimerx[eq_]:=Module[{function,functionG,functionNH,functionKprime},
```

(*Derives the functions of pH and ionic strength that give the standard transformed Gibbs energy of reaction, change in number of hydrogen ions bound, and the apparent equilibrium constant at 298.15 K for a biochemical reaction typed in as, for example, $\text{atp} + \text{h}_2\text{o} + \text{de} = \text{adp} + \text{pi}$. The standard transformed Gibbs energy of reaction is in kJ mol^{-1} .*)

```
function=Solve[eq,de];
functionG=function[[1,1,2]];
functionNH=(1/(8.31451*.29815*Log[10]))*D[functionG,pH];
functionKprime=Exp[-functionG/(8.31451*.29815)];
{functionG,functionNH,functionKprime}];
```

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